Philipps Universität Marburg

# Ökologie methanotropher Bakterien:

Räumliche Verteilung und Funktion methanotropher Bakterien in Feuchtgebieten

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„Aufgabe der Naturwissenschaft ist es nicht
nur die Erfahrung zu erweitern, sondern in
diese Erfahrung eine Ordnung zu bringen.“

Niels Bohr, Physiker

# Zusammenfassung

Methan ist neben $CO_2$ das wichtigste Treibhausgas, dessen relatives Treibhauspotential ungefähr ein drittel höher liegt als das von $CO_2$. Der Großteil atmosphärischen Methans wird dabei aus biogenen Methanquellen freigesetzt, zum Beispiel renaturierte Mülldeponien, Feuchtgebiete oder Reisfelder. Methanotrophe Bakterien (MOB) können die Methanemission hier um bis zu 80 % reduzieren. Infolgedessen ist ihre Physiologie, Diversität und Ökologie in zahlreichen Studien untersucht worden. Es fehlen jedoch grundlegende Studien über die räumliche Verteilung von MOB in ihrer Umwelt. Des Weiteren sind die Populationsdynamiken von MOB und die Beteiligung spezifischer Taxa an der Methanoxidation bisher wenig verstanden. Zudem beginnt man erst jetzt zu erkennen, dass Umweltstörungen einen signifikanten Effekt auf die Stabilität und Funktion mikrobieller Lebensgemeinschaften haben. Die Zusammenhänge von Diversität und Funktion und die Regulation der MOB durch natürliche und/oder anthropogene Umweltfaktoren sind bisher jedoch kaum untersucht worden.

In dieser Arbeit wurde das *pmoA* Gen als phylogenetischer und funktioneller Marker verwendet, um MOB in Umweltproben zu detektieren. Während es speziell an das Reisfeld adaptierte *pmoA* Genotypen zu geben scheint, können sich methanotrophe Lebensgemeinschaften in Reisfeldern derselben Region deutlich unterscheiden. Der Einfluss von Umweltgradienten variiert in Agrar- und natürlichen Ökosystemen und muss bei der Planung von Experimenten berücksichtigt werden. Am Beispiel von Reisfeldern konnte gezeigt werden, dass MOB keine großskalige räumliche Strukturierung aufwiesen und sowohl eine systematische als auch eine Zufallsprobennahme repräsentativ ist. Zudem konnten Populationdynamiken nach der Flutung eines Reisfeldes nachgewiesen werden, obwohl die Methanoxidationrate konstant blieb. Eine artenreiche mikrobielle „seed bank“ scheint für die Erhaltung der Funktion in solchen dynamischen Ökosystemen eine große Rolle zu spielen. Betrachtet man sich die methanotrophe Lebensgemeinschaft unter verschieden Energieflüssen und dem Effekt von Stickstoffdüngung, so hat die Düngung keinen Effekt auf die methanotrophen Lebensgemeinschaften. Es werden jedoch unter verschiedenen Energieflüssen aus der „seed bank“ unterschiedliche MOB aktiviert. Es scheint, dass Arten der Gattung *Methylobacter* und Arten deren *pmoA* Sequenzen zu einem Cluster

mit Umweltsequenzen aus Reisfeldern gehören, speziell an Habitate mit hoher Methankonzentration adaptiert sind. MOB scheinen sehr widerstandsfähig zu sein und Änderungen in Energieflüssen scheinen einen größeren Effekt auf die methanotrophe Lebensgemeinschaft zu haben.

## Summary

Methane is the second most important greenhouse gas after $CO_2$ exerting a radiative forcing about a third of that of $CO_2$. Most of the atmospheric methane is released from biogenic sources such as landfills, natural wetlands and rice fields. Methane emission from these sources would be significantly higher without the activity of methanotrophs that oxidize the biogenically produced methane, thus reducing the methane emissions up to 80 %. Consequently, the physiology, diversity and ecology of methanotrophs have been studied. However, influences of biogeographical patterns and spatial heterogeneities on the methanotrophic community are poorly investigated. Furthermore, little is known about population dynamics and contribution of specific taxa to methane oxidation. The effect of environmental disturbances on the stability and function of microbial communities has just begun to be realized. However, a link between diversity and function and the regulation of methanotrophic communities by natural and/or anthropogenic factors are not known in detail.

In this thesis the *pmoA* gene was used as a functional and phylogenetic marker for the identification of methanotrophs from environmental samples. On a global scale certain *pmoA* genotypes seem to be specifically adapted to paddy fields while at closely geographically located field sites methanotrophic communities revealed different community patterns. The influence of environmental gradients varies between different habitats and has to be considered when designing experimental studies. In the studied agroecosystem, population structure showed no spatial pattern implying that both a systematic and random sampling design would be adequate. We observed a succession of methanotrophs, however, the oxidation performance stayed relatively stable. Hence, a diverse microbial seed bank of methanotrophs seems to play an important role in maintaining the function in such a dynamic ecosystem. From this seed bank different methanotrophs are activated under high and low energy fluxes. We identified species of the genus *Methylobacter* and an environmental cluster strictly affiliated with paddy soils that seem to be adapted to high methane environments. Methanotrophic community was not significantly affected by nitrogen fertilization under different energy flows. We suggest that methanotrophs are quite resilient, and that changes in the energy flow have major effects for the community structure.

# 1. Einleitung

## 1.1 Methan als Treibhausgas in der Atmosphäre

Methan ($CH_4$), als einfachster Vertreter der Kohlenwasserstoffe, gilt neben Kohlendioxid ($CO_2$) als wichtigstes Treibhausgas (Cicerone and Oremland, 1988) und besitzt ein 21fach höheres relatives Treibhauspotential als $CO_2$. Seit der vorindustriellen Zeit ist die Methankonzentration bis 2005 kontinuierlich von etwa 715 ppb auf etwa 1774 ppb gestiegen (Forster et al., 2007). Untersuchungen an Eisbohrkernen belegen, dass der Anteil an $CH_4$ in der Atmosphäre in den letzten 650.000 Jahren (400 ppb bis 700 ppb) noch nie so stark gestiegen ist wie derzeit (Spahni et al., 2005). Die jährliche Zunahme an $CH_4$ in der Atmosphäre sank kontinuierlich von 14 ppb in den 80er Jahren bis zum Ende der 90er Jahre auf beinahe Null. Seitdem hat sie sich nicht mehr wesentlich verändert (Dlugokencky et al., 1998; Dlugokencky et al., 2003). Die Ursachen dieser kontinuierlichen Abnahme sind kontrovers diskutiert worden (Dlugokencky et al., 1998; Lelieveld et al., 1998; Hansen et al., 2000). Erst kürzlich wurde ein Wiederanstieg der jährliche Zunahme an $CH_4$ in der Atmosphäre gezeigt (Rigby et al., 2008). Des Weiteren gibt es Hinweise auf große Fluktuationen von Methanquellen und Methansenken, die einen Wiederanstieg der jährlichen Zunahme an $CH_4$ in der Atmosphäre bewirken können (Dlugokencky et al., 1998; Bousquet et al., 2006).

Die jährliche Methanemission in die Atmosphäre aus allen derzeit bekannten Quellen liegt im Durchschnitt zwischen 500 und 600 Tg (Dlugokencky et al., 1998; Denman et al., 2007; Conrad, 2009). Ob es zu einem Anstieg oder einer Abnahme der Methankonzentration in der Atmosphäre kommt ergibt sich aus der Bilanz von Methanquellen und Methansenken. Die Methanquellen lassen sich allgemein zwischen natürlichen und anthropogen beeinflusste Quellen unterscheiden, wobei der Anteil anthropogen beeinflusster Quellen deutlich höher ist (Abb. 1). Mehr als 60 % des produzierten $CH_4$ sind das Ergebnis mikrobiologischer Aktivität. Das sogenannte biogene $CH_4$ wird während der anaeroben Zersetzung organischen Materials von methanogenen Archaea vorwiegend aus $CO_2$ und Wasserstoff oder $CO_2$ und Acetat gebildet (Conrad, 2007).

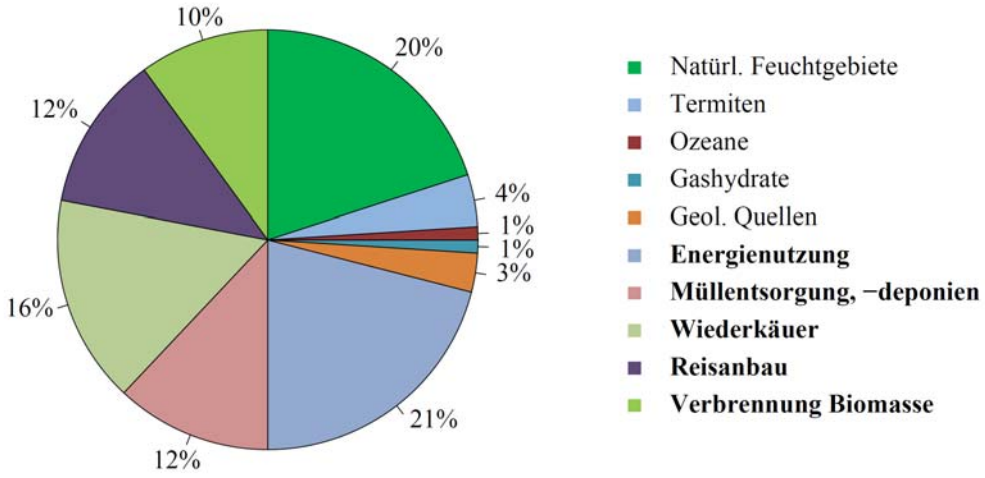


**Abb. 1:** Prozentualer Anteil der einzelnen Methanquellen an der jährlichen $CH_4$ Produktion (Denman et al., 2007); Anthropogen beeinflusste Quellen sind fettgedruckt.

Erst kürzlich gab es Hinweise auf eine zusätzliche Methanquelle. Hierbei handelt es sich um die chemische Produktion von Methan in Pflanzenblättern (Keppler et al., 2006). Diese Ergebnisse werden jedoch kontrovers diskutiert, da andere Wissenschaftler diese Ergebnisse nicht reproduzieren konnten (Dueck et al., 2007; Beerling et al., 2008). Indes wurde die chemische Methanproduktion aus Blattmaterial und Pektin durch UV-Strahlung mehrfach nachgewiesen (McLeod et al., 2008; Vigano et al., 2008).

Die Troposphäre stellt mit einem Anteil von 90 % die größte Methansenke dar. Durch photochemische Oxidation reagiert $CH_4$ mit Hydroxyl-Radikalen, wobei hauptsächlich Wasser ($H_2O$) und $CO_2$ freigesetzt werden. Daneben diffundiert $CH_4$ in die Stratosphäre. Die letzte bekannte Methansenke umfasst einen ausschließlich mikrobiologischen Prozess, bei dem eine Gruppe von Bakterien, die sogenannten methanotrophen Bakterien (MOB), atmosphärisches $CH_4$ oxidieren (Bender and Conrad, 1992; Knief et al., 2003).

## 1.2 Die Bedeutung von Reisfeldern als Methanquelle

Reis gilt als eines der wichtigsten Grundnahrungsmittel und wird weltweit auf einer Fläche von ungefähr 1.5 Millionen km² angebaut (Fernando, 1993; Minami and Neue, 1994). Man unterscheidet zwischen Trocken- und Nassreisanbau. Der Nassreisanbau wird weiter unterteilt in regengefluteten, saisonal künstlich gefluteten und permanent

gefluteten Anbau. Derzeit werden 50 % der Fläche in Form von Nassreisanbau bewirtschaftet. Dieser Anteil entspricht 70 % der weltweiten Reisproduktion (Neue and Roger, 2003).

Als Methanquelle spielt nur der Nassreisanbau eine wesentliche Rolle. Die jährlichen Methanemissionen liegen hier zwischen 20 und 110 Tg $a^{-1}$ (Cicerone and Oremland, 1988; Denman et al., 2007). Dies entspricht je nach Berechnungsgrundlage einem Anteil von bis zu 18 % der jährlichen globalen Methanemissionen. Damit stellt der Nassreisanbau eine der größten Einzelquellen an $CH_4$ dar (Denman et al., 2007). Seit den 60er Jahren hat sich die Reisproduktion von ungefähr 240 Tt $a^{-1}$ auf ungefähr 650 Tt $a^{-1}$ fast verdreifacht (IRRI, 2010). Um eine Versorgung der stetig wachsenden Weltbevölkerung mit Grundnahrungsmitteln zu gewährleisten, muss die Reisproduktion weiter gesteigert werden. Die wachsende Nachfrage kann vermutlich nur durch eine Intensivierung des Nassreisanbaus und eine Erweiterung der bestehenden Reisanbaugebiete erreicht werden (Neue and Roger, 2003). Diese Maßnahmen könnten zu einem Anstieg der Methanemissionen führen.

Genau hier kommt den MOB eine Schlüsselrolle zu, denn neben der Bedeutung als Methansenke haben diese Organismen einen wesentlichen Einfluss auf die Emission von $CH_4$ aus biogenen Methanquellen. Sie oxidieren $CH_4$ zu $CO_2$ und $H_2O$ bevor es in die Atmosphäre gelangt (Frenzel, 2000; Reeburgh, 2003). Als sogenannte Biofilter können methanotrophe Bakterien die potentiellen Methanemissionen von Nassreisfeldern um bis zu 80 % reduzieren (Conrad and Rothfuss, 1991).

## 1.3 Das Agrarökosystem Reisfeld

Reisfelder stellen ein differenziertes Ökosystem dar. Sobald ein Reisfeld geflutet wird, verbrauchen aerobe Bakterien den restlichen Sauerstoff im Boden und es bilden sich verschiedene Bereiche mit unterschiedlichen physico-chemischen Eigenschaften aus. Das Agrarökosystem Reisfeld kann in drei große mikrobielle Habitate unterteilt werden (Liesack et al., 2000):

- dem oxisch/anoxischen Grenzbereich zwischen Flutwasser und Boden
- den anoxischen Boden
- die Rhizosphäre

Den größten Bereich macht der anoxische Boden aus. Dort werden organische Verbindungen durch verschiedene anaerobe Prozesse mikrobiell abgebaut. Zunächst

findet gemäß der Thermodynamik eine sequentielle Reduktion der alternativen Elektronenakzeptoren $NO_3^-$, Mn(IV), Fe(III), $SO_4^{2-}$ und $CO_2$ statt (Ponnamperuma, 1972; Zehnder and Stumm, 1988). Diese werden durch Reoxidation in der oxisch/anoxischen Grenzschicht sowie der Rhizosphäre wieder regeneriert (Conrad and Frenzel, 2002). In Abwesenheit von Sauerstoff und alternativen Elektronenakzeptoren wandeln methanogene Archaea organischen Kohlenstoff zu $CO_2$ und $CH_4$ um. Der organische Kohlenstoff stammt zu 80 % aus abgebautem Reisstroh, sowie aus Photosyntheseprodukten, die durch die Wurzel ausgeschieden werden, und zu einem Teil aus abgestorbenem Wurzelmaterial (Watanabe et al., 1999).

Das produzierte $CH_4$ bildet das Substrat für die Methanoxidation durch aerobe MOB. Diese findet man typischerweise an der oxisch/anoxischen Grenzschicht, wo Methan- und Sauerstoffgradienten überlappen. Die Rhizosphäre stellt ein weiteres ökologisch wichtiges Habitat für die MOB dar, denn Reis besitzt wie alle Hygrophyten ein ausgeprägtes Durchlüftungsgewebe (Aerenchym), das im allgemeinen Gasaustausch $O_2$ über die Wurzeln in die Rhizosphäre transportiert und somit für die Methanoxidation zur Verfügung stellt (Bosse and Frenzel, 1997).

## 1.4 Methanotrophe Bakterien

*Aerobe methanotrophe Bakterien*

Die aeroben MOB zählen zu den methylotrophen Mikroorganismen und bilden eine physiologisch eigenständige Gruppe. Während die methylotrophen Bakterien eine Vielzahl an C1-Verbindungen als Energie- und Kohlenstoffquelle nutzen, sind MOB die einzigen Bakterien, die $CH_4$ verwenden können. Neben $CH_4$ verwerten MOB aber auch Methanol, Methylamin und einige wenige andere C1-Verbindungen als Substrat (Bowman, 2006). Bisher wurde angenommen, dass MOB obligat methylotroph sind. Es konnte jedoch auch fakultative Methylotrophie gezeigt werden, so kann der Stamm *Methylocella silvestris* BL2 neben Methan und Methanol auch zum Beispiel Acetat verwerten (Dedysh et al., 2005; Conrad, 2009).

MOB oxidieren $CH_4$ über Methanol, Formaldehyd und Formiat zu $CO_2$. Zum Aufbau von Biomasse wird Kohlenstoff aus dem Formaldehyd über zwei unterschiedliche Stoffwechselwege, den Ribulose-Monophosphat-Weg oder den Serin-Weg assimiliert. Der erste Schritt bei der Methanoxidation wird durch zwei verschiedene Typen des Enzyms Methan-Monooxygenase (MMO) katalysiert. Während

die lösliche, zytoplasmatische MMO (sMMO) nur in wenigen MOB vorhanden ist, kann man die partikuläre, membrangebundene MMO (pMMO), außer in *Methylocella palustris*, in allen bekannten MOB finden (Theisen et al., 2005). In Organismen, die beide Enzyme besitzen, wird die sMMO nur unter geringen Kupferkonzentrationen exprimiert (Nielsen et al., 1996). Das *pmoA* Gen, welches eine Untereinheit der pMMO codiert, hat sich als hervorragender phylogenetischer und funktioneller Marker für die Identifizierung von MOB aus Umweltproben erwiesen (McDonald and Murrell, 1997; McDonald et al., 2008).

Innerhalb der MOB wird zwischen zwei großen Gruppen unterschieden, Typ I und II: Typ I MOB gehören zu den γ-Proteobakterien während Typ II MOB den α-Proteobakterien zugeordnet werden. Basierend auf der Phylogenie werden die Typ I MOB weiter unterteilt in Typ Ia und Typ Ib (Bodrossy et al., 2003). Typ Ib enthalten neben einer Reihe unkultivierter Vertreter die Gattungen *Methylococcus* und *Methylocaldum* und wurden in früheren Veröffentlichungen als Typ X bezeichnet (Bowman, 2006). Typ Ia enthält viele kultivierte Vertreter, wie zum Beispiel *Methylobacter*, *Methylomonas*, *Methylosarcina* und zahlreiche Umweltklone (Bodrossy et al., 2003). Erst vor kurzem wurde die Methanotrophie auch bei *Crenothrix polyspora*, *Clonotrhrix fusca* und einigen Stämmen der Verrucomicrobia entdeckt (Stoecker et al., 2006; Dunfield et al., 2007; Vigliotta et al., 2007; Islam et al., 2008). Die Proteobakterien wurden jedoch weitaus länger untersucht. Die wichtigsten Charakteristika der MOB sind in Tabelle 1 zusammengefasst. Die klassische Einteilung in Typ I und II basiert auf der Phylogenie, der unterschiedlichen Kohlenstoff-Fixierung, der Anordnung interner Membranen, der Fähigkeit Stickstoff zu fixieren und der unterschiedlichen Phospholipid-Fettsäuren (PLFA) (Hanson and Hanson, 1996; Conrad, 2007). Die Entdeckung neuer Arten hat jedoch gezeigt, dass diese biochemische und morphologische Einteilung nicht allgemeingültig ist, zum Beispiel *Methylocystis heyeri*, der sowohl PLFA der Typ I als auch der Typ II besitzt (Dedysh et al., 2007). Ein weiteres Beispiel ist die Fähigkeit der Stickstoff-Fixierung, die lange Zeit nur den Typ II MOB zugeschrieben wurde, aber auch in Typ I nachgewiesen werden konnte (Auman et al., 2001). Obwohl die klassische Einteilung in Typ I und Typ II MOB nicht mehr allgemeingültig ist, wird sie weiterhin für die Unterscheidung der drei Familien *Methlyococcaeae* (Typ I), *Methylocystaceae* und *Beijerinckiaceae* (Typ II) verwendet.

Die Ammoniak oxidierenden Bakterien (AOB) können neben Ammoniak auch Methan zu Methanol umwandeln, wobei die Bedeutung für die globalen

Methanemissionen als gering eingeschätzt werden (Bedard and Knowles, 1989; Bodelier and Frenzel, 1999). Der erste Schritt der aeroben Nitrifikation, die Umwandlung von Ammoniak zu Hydroxylamin, wird durch die Ammoniak-Monooxygenase (*amoA*) katalysiert. Das *amoA* Gen ist homolog zum *pmoA* Gen und deshalb wird zum Teil das *amoA* Gen der AOB mit einigen *pmoA* spezifischen PCR Assays mitamplifiziert (Holmes et al., 1995).

**Tabelle 1: Zusammenfassung typischer Charakteristika von Typ I, Typ II und Verrucomicrobia (Dedysh et al., 2000; Dedysh et al., 2002; Dunfield et al., 2003; Tsubota et al., 2005; Bowman, 2006; Rahalkar et al., 2007; Hou et al., 2008; Chen and Murrell, 2009).**

| Typ | Phylogenie | Familie | Gattung | pMMO | sMMO | $C_1$-Ass.[1] | ICM[2] | $N_2$ fix.[3] | PLFA[4] | Dauerstadien |
|---|---|---|---|---|---|---|---|---|---|---|
| Typ Ia | γ-Proteobakterien | *Methylococcaecae* | *Methylomonas* | + | +/- | RumP[5] | Typ I | +/- | 16:01 | Zysten[7] |
| Typ Ia | | | *Methylobacter* | + | - | RumP[5] | Typ I | - | 16:01 | Zysten[7] |
| Typ Ia | | | *Methylomicrobium* | + | +/- | RumP[5] | Typ I | - | 16:01 | Keine |
| Typ Ia | | | *Methylosarcina* | + | - | RumP[5] | Typ I | - | 16:01 | Variabel |
| Typ Ia | | | *Methylosphaera* | + | - | RumP[5] | nd[6] | + | 16:01 | Keine |
| Typ Ia | | | *Methylosoma* | + | - | unbekannt | Typ I | + | 16:01 | Zysten |
| Typ Ia | | | *Methylothermus* | + | - | RumP[5] | Typ I | - | 18:1/16:0 | Keine |
| Typ Ia | | | *Methylohalobius* | + | - | RumP[5] | Typ I | - | 18:01 | Keine |
| Typ Ib | γ-Proteobakterien | *Methylococcaecae* | *Methylococcus* | + | + | RumP[5]/Serine | Typ I | + | 16:01 | Zysten[7] |
| Typ Ib | | | *Methylocaldum* | + | - | RumP[5]/Serine | Typ I | - | 16:01 | Zysten[7] |
| (Typ I) | | | *Crenothrix* | + | - | nd[6] | Typ I | nd[6] | nd[6] | nd[6] |
| (Typ I) | | | *Clonothrix* | + | - | nd[6] | Typ I | nd[6] | nd[6] | nd[6] |
| Typ II | α-Proteobakterien | *Methylococcaecae* | *Methylosinus* | + | + | Serine | Typ II | + | 18:01 | Exosporen |
| Typ II | | | *Methylocystis* | + | +/- | Serine | Typ II | + | 18:01 | Lipid-Zysten |
| Typ II | | *Beijerinckiaceae* | *Methylocella* | - | + | Serine | nd | + | 18:01 | Keine/Exosporen |
| Typ II | | | *Methylocapsa* | + | - | Serine | Typ III | + | 18:01 | Zysten[7] |
| - | Verrucomicrobia | *Verrucomicrobiaceae* | *Methylacidiphilum* | + | - | Serine ? | nd[6] | - | C14-C15, C18 | ? |

[1] Kohlenstoff-Fixierung [2]Intrazytoplasmatische Mebranen: Stapelförmig (Typ I), entlang der Zytoplasmamembran (Typ II), parallel zur Zytoplasmamembran auf einer Seite der Zelle (Typ III) [3]Stickstoffs-Fixierung [4] Charakteristische Phospholipid-Fettsäuren [5] Ribulose-Monophosphat-Weg [6] nicht definert [7] *Azotobacter* ähnlich

*Aerobe methanotrophe Bakterien in „Upland soils“*

Die Oxidation von atmosphärischem $CH_4$ stellt in Böden eine wichtige Methansenke dar. Hier wird $CH_4$ unter atmosphärischen Konzentrationen, mit einem $K_{m(app)}$ Wert im nanomolaren Bereich oxidiert (Bender and Conrad, 1992). Alle damals bekannten kultivierten Vertreter der MOB wiesen jedoch einen $K_{m(app)}$ Wert von >1µMolar auf (Bedard and Knowles, 1989). Bisher gibt es keine kultivierten Vertreter dieser sogenannten „high affinity“ MOB, aber es gibt einige Kandidaten, die für die Methanoxidation unter atmosphärischen Konzentrationen in Frage kommen (Holmes et al., 1999; Knief et al., 2003).

In einigen *Methylosinus* und *Methylocystis* Arten konnten zwei Formen der pMMO nachgewiesen werden, die konventionelle „low affinity“ pMMO und eine neue „high affinity“ pMMO, die zum Wachstum unter atmosphärischer Methankonzentration befähigt (Dunfield et al., 2002; Ricke et al., 2004). Ein Wachstum unter atmosphärischer Methankonzentration konnte bisher nur für den Stamm *Methylocystis* SC2 gezeigt werden (Baani and Liesack, 2008). Ob diese Mikroorganismen für die atmosphärische Methanoxidation verantwortlich sind konnte bisher nicht geklärt werden.

*Anaerobe Methanoxidation*

Zurzeit geht man davon aus, dass bei der anaeroben Methanoxidation $CH_4$ durch die Reduktion von Sulfat oxidiert wird. Dieser Prozess findet vermutlich in einer syntrophen Beziehung zwischen $CH_4$-oxidierenden, Wasserstoff ($H_2$)-produzierenden Archaea und $H_2$-verbrauchenden, Sulfat-reduzierenden Bakterien statt (Hoehler et al., 1994; Krüger et al., 2008; Thauer and Shima, 2008). Alle derzeit bekannten anaeroben Methanoxidierer gehören zu den methanogenen Euryarchaeota, jedoch ohne kultivierte Vertreter. Man unterscheidet drei phylogenetische Cluster von methanotrophen Archaea: ANME-1, ANME-2 und ANME-3 (Knittel and Boetius, 2009). Diese Cluster sind in marinen Habitaten weit verbreitet, konnten bisher aber nicht in Reisfeldern detektiert werden (Conrad, 2007).

Neben Sulfat könnten theoretisch auch andere Elektronen-Akzeptoren mit einer höheren Energieausbeute genutzt werden. Erste Studien belegen die Kopplung der anaeroben Methanoxidation mit Nitrat, Fe(III) oder Mn (IV) (Beal et al., 2009; Ettwig et al., 2009). Hinweise auf eine anaerobe Methanoxidation mit Fe(III) in tieferen Schichten von Reisfeldern wurden nicht weiter verfolgt (Murase and Kimura, 1994a;

Murase and Kimura, 1994b). Nach derzeitigem Kenntnisstand hat die anaerobe Methanoxidation durch anaerobe methanotrophe Archaea (ANME) ihre größte Bedeutung in den Ozeanen (Knittel and Boetius, 2009).

## 1.5 Ökologische Differenzierung methanotropher Bakterien

Über die ökologische Differenzierung methanotropher Bakterien ist bisher wenig bekannt. Ausnahmen bilden nur Vertreter aus extremen Habitaten, wie zum Beispiel die halophile Art *Methylohalobius crimeensis* (Heyer et al., 2005).

Es wurden jedoch einige Hypothesen formuliert, die sich wie folgt zusammenfassen lassen: Eine Hypothese besagt, dass Typ I MOB geringe Methan- und hohe Sauerstoffkonzentrationen und Typ II MOB vorwiegend hohe Methan- und geringe Sauerstoffkonzentrationen bevorzugen (Graham et al., 1993; Amaral and Knowles, 1995). Am Beispiel des Reisfeldes ergab sich daraus später die Theorie, dass Typ I und II unterschiedliche Nischen besetzen und koexistieren. Dabei dominieren Typ II MOB in gefluteten Reisfeldern, wenn die Methanverfügbarkeit hoch ist und die Umweltbedingungen ausgeglichen sind. Im Gegensatz dazu sind Typ I MOB eher in trockengelegten Reisfeldern vorherrschend, wenn die Methanverfügbarkeit niedrig ist und die Umweltbedingungen sich rasch verändern (Henckel et al., 2000). Am Beispiel von hydromorphen Böden konnte jedoch gezeigt werden, dass unter atmosphärischen Methankonzentrationen die dominierenden PLFAs zu den Typ II MOB gehörten, während unter hohen Methankonzentration zusätzlich Typ I MOB typische PLFAs nachgewiesen wurden (Knief et al., 2006). Eine weitere Theorie basierte darauf, dass Typ II MOB im Gegensatz zu Typ I MOB Stickstofffixierer sind (Graham et al., 1993; Hanson and Hanson, 1996). So konnte gezeigt werden, dass Typ I MOB in der Reis-Rhizosphäre durch Ammonium-Düngung mehr stimuliert wurden als Typ II MOB (Bodelier et al., 2000). Die Entdeckung der Stickstoff-Fixierung bei einigen Typ I MOB stellt jedoch diese Annahme in Frage (Auman et al., 2001).

Hieraus wird deutlich, dass es kein grundlegendes Verständnis zu den ökologischen Unterschieden zwischen Typ I und II MOB gibt. Zudem fehlt ein ausführliches Wissen über den Zusammenhang methanotropher Lebensgemeinschaft mit Umweltfaktoren.

## 1.6 Die Biogeographie methanotropher Bakterien

Das Verständnis der Entwicklung und Aufrechterhaltung der Biodiversität ist ein grundlegendes Ziel der Ökologie (Green and Bohannan, 2006). Hier bekommt die Biogeographie eine zentrale Bedeutung, denn sie versucht räumliche Verteilungsmuster der Biodiversität und die Veränderung dieser Muster über die Zeit zu beschreiben und zu erklären (Fierer, 2008). In vielen Diversitätsstudien von Pflanzen und Tieren konnten biogeographische Verteilungsmuster nachgewiesen werden. Nur wenige Studien konzentrieren sich auf Mikroorganismen (Green and Bohannan, 2006; Hughes Martiny et al., 2006), denn es ist schwierig mikrobielle Diversität zu erfassen. Erst die Entwicklung und Anwendung molekularbiologischer Methoden, zum Beispiel „Sanger sequencing", zeigten, dass frühere kultivierungsabhängige Studien nur einen Teil der mikrobiellen Biodiversität erfasst haben (Head et al., 1998). Auf der anderen Seite hat man lange Zeit angenommen, dass Mikroorganismen sich so deutlich von den übrigen Lebensformen unterscheiden, dass ihre Biodiversität grundsätzlich anders skaliert ist (Green and Bohannan, 2006). Lourens G.M. Bass Becking stellte bereits 1934 die Hypothese auf (zusammengefasst in: de Wit and Bouvier, 2006; O'Malley, 2007): "*Everything is everywhere, but the environment selects.*", das heißt Mikroorganismen sind omnipräsent und entwickeln sich in jedem Habitat mit geeigneten Umweltbedingungen (Green et al., 2008). In neuerer Zeit häufen sich aber Beispiele mikrobiellen Endemismus und räumlicher Strukturierung mikrobieller Diversität (Whitaker et al., 2003; Bell et al., 2005). Durch dieses Umdenken ist die mikrobielle Biogeographie in den letzten Jahren in den Fokus des Interesses gerückt (Fierer and Jackson, 2006; Hughes Martiny et al., 2006; Ramette and Tiedje, 2007; Green et al., 2008).

Die räumliche Verteilung scheint ein wesentlicher Faktor zu sein, der Artendynamiken und Biodiversität vieler Lebensgemeinschaften bestimmt. Im Boden hängt die räumliche Verteilung der bakteriellen Diversität teilweise von einem breiten Spektrum an bodenphysikalischen Strukturen ab (Grundmann, 2004). So wurde zum Beispiel vorgeschlagen, dass Bodenaggregate ein besonderes Mikrohabitat darstellen, das für bestimmte Bakteriengruppen selektiert (Mummey et al., 2006). Böden sind zudem chemisch sehr komplex durch zum Beispiel steile Gradienten der Substratkonzentrationen, Redox Potential und pH Wert und sind somit an der Bildung vieler Mikrohabitate beteiligt. Dies erhöht die Möglichkeit der Spezialisierung und

Auftrennung in deutliche ökologisch unterscheidbare Arten (Torsvik et al., 2002). Die Einflüsse biotischer und abiotischer Faktoren, die für eine beobachtete räumliche Strukturierung der mikrobiellen Lebensgemeinschaften verantwortlich sein können, sind allerdings bisher wenig verstanden (Fierer et al., 2009).

Die Biogeographie methanotropher Bakterien ist bisher kaum untersucht worden. In einer Studie über die räumliche Verteilung MOB in der deckenden Bodenschicht einer stillgelegten Mülldeponie wurde gezeigt, dass auf einer Skala von 5 m methanotrophe Lebensgemeinschaften keine erkennbaren Muster aufwiesen (Kumaresan et al., 2009). Auch für MOB in Reisfeldern wurden bisher keine biogeographischen Muster nachgewiesen, obwohl Vertreter der Gattung *Methylocaldum* hierbei tendenziell nur in tropischen Regionen detektiert wurden (Chen and Murrell, 2009).

## 1.7 Ziele der Arbeit

Methanotrophe Bakterien spielen eine Schlüsselrolle für die globale Methanemission. Sie stellen die einzige biologische Methansenke dar und haben die größte Bedeutung in ihrer Funktion als Biofilter in Habitaten mit hohen Methankonzentrationen. Infolgedessen ist ihre Physiologie, Diversität und Ökologie sehr genau untersucht worden (Hanson and Hanson, 1996; Conrad, 2007; Trotsenko and Murrell, 2008; Conrad, 2009). Molekularbiologische Methoden erlauben eine differenzierte Analyse der MOB (McDonald et al., 2008). Hinsichtlich ihrer Ökologie fehlen jedoch Untersuchungen, über räumliche Verteilungsmuster, Zusammenhänge von Diversität und Funktion und die Regulation der MOB durch natürliche und/oder anthropogene Umweltfaktoren. Dieses Wissen ist jedoch wichtig, um die beobachteten Dynamiken in den globalen Methanemissionen zu verstehen.

In dieser Arbeit wurde das *pmoA* Gen als phylogenetischer und funktioneller Marker verwendet. Es wurden verschiedene Fingerprint-Methoden in Kombination mit multivariaten statistischen Analysen genutzt um die Ökologie methanotropher Lebensgemeinschaften zu entschlüsseln.

*Kapitel 2*

Hier werden MOB als Modellsystem verwendet, um mikrobielle Biogeographie zu untersuchen. Die zentralen Fragen lauten, ob MOB biogeographische Muster erkennen lassen und wenn ja, auf welchen Ebene diese auftreten.

*Kapitel 3 und 4*

Reisfelder sind Agrarökosysteme, welche sehr homogen sind. Es wird der Frage nachgegangen, ob methanotrophe Lebensgemeinschaften lokal räumlich strukturiert sind. Als Beispiel für ein natürliches heterogenes Ökosystem wird im Vergleich das Littoral eines borealen Sees untersucht.

*Kapitel 5*

Dieses Kapitel beschäftigt sich mit der Sukzession der methanotrophen Lebensgemeinschaft. Dabei sollen in einem Modellsystem die Fragen beantwortet werden, welche MOB an der Sukzession nach der Flutung von Reisfeldern beteiligt sind, welche physiologisch aktiv sind und ob es umweltbedingte Faktoren gibt, die die Sukzession MOB beeinflussen.

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# 2. Biogeography of wetland rice methanotrophs

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## 2.1 Abstract

We focused on the functional guild of methane oxidizing bacteria (MOB) as model organisms to get deeper insights into microbial biogeography. The *pmoA* gene was used as a functional and phylogenetic marker for MOB in two approaches: (i) a *pmoA* database (> 4000 sequences) was evaluated to obtain insights into MOB diversity in Italian rice paddies, and paddy fields worldwide. The results show a wide geographical distribution of *pmoA* genotypes that seem to be specifically adapted to paddy fields (e.g. Rice Paddy Cluster 1 and Rice Paddy Cluster 2). (ii) On the smaller geographical scale, we designed a factorial experiment including three different locations, two rice varieties and two habitats (soil and roots) within each of three rice fields. Multivariate analysis of terminal restriction fragment analysis profiles revealed different community patterns at the three field sites, located 10–20 km apart. Root samples were characterized by high abundance of type I MOB whereas the rice variety had no effect. With the agronomical practice being nearly identical, historical contingencies might be responsible for the field site differences. Considering a large reservoir of viable yet inactive MOB cells acting as a microbial seed bank, environmental conditions might have selected and activated a different subset at a time thereby shaping the community.

## 2.2 Introduction

Next to $CO_2$, methane is the most important greenhouse gas contributing substantially to radiative forcing (Intergovernmental Panel on Climate Change, 2007). Natural wetlands and rice fields belong to the major sources of atmospheric $CH_4$ (Intergovernmental Panel on Climate Change, 2007). In contrast, upland soils function as a sink to atmospheric $CH_4$ due to the uptake by methane oxidizing bacteria (MOB) (Conrad, 1996; Knief et al., 2003). On a global scale, however, MOB are even more important in wetlands, where they function as a bio-filter preventing $CH_4$ produced in anoxic layers escaping into the atmosphere. The MOB in natural wetlands and rice paddies attenuate the potential $CH_4$ emission by up to 40 % (Reeburgh et al., 1993; Frenzel, 2000). In particular situations, e.g. at the oxic-anoxic boundary near the very surface of sediments or water-saturated soils, an attenuation of more than 90 % may be achieved (Frenzel et al., 1990; Gilbert and Frenzel, 1998). Another oxic-anoxic boundary is formed in the rhizosphere of wetland plants, where $O_2$ diffusing through the aerenchyma may be released from the roots supporting root-associated $CH_4$ oxidation (Armstrong, 1971; Conrad and Frenzel, 2002). In rice fields, root-associated $CH_4$ oxidation is the most important $CH_4$ sink. A couple of case studies in wetland rice fields have reported on $CH_4$ oxidation rates of 10–30 % per season (Denier van der Gon and Neue, 1996; Krüger et al., 2002; Eller et al., 2005). However, considerable differences emerge comparing studies on different rice cultivars (Denier van der Gon and Neue, 1996; Tyler et al., 1997; Bosse and Frenzel, 1998; Bilek et al., 1999; Marik et al., 2002; Eller et al., 2005): $CH_4$ oxidation may occur more or less throughout the season, decline with the onset of the reproductive period, but sometimes become re-established at the very end of the season. Only part of this pattern can be attributed to agricultural practice like fertilization with mineral nitrogen (Bodelier et al., 2000a, b): this effect tends to be transient (Krüger et al., 2002) becoming unimportant late in the season (Dan et al., 2001). How the populations of MOB growing on and in rice roots are affected by cultivars, and how in turn they may affect emissions, is largely unknown.

Basically, two types of MOB are distinguished: type I and type II corresponding to the families *Methylococcaceae* (type I, γ-*Proteobacteria*), *Methylocystaceae* and *Bejerinckiaceae* (type II, α-*Proteobacteria*) (Bowman, 2000). This classification was originally based on phenotypical traits, but corresponds well to the phylogeny of the *pmoA* gene encoding the α-subunit of the particulate methane monooxygenase. Type I

MOB can be further divided into type Ia comprising the genera *Methylomonas*, *Methylobacter*, *Methylosoma*, *Methylosarcina* and *Methylomicrobium*, and type Ib characterized by *Methylococcus* and *Methylocaldum*. Type Ib was previously also referred to as type X. Recently, MOB belonging to the phylum *Verrucomicrobia* were isolated; however, they seem to be restricted to extreme environments (e.g. Dunfield et al., 2007). *pmoA* was found to be an excellent functional marker (McDonald and Murrell, 1997) becoming the most frequently used target in molecular ecology studies of MOB (Dumont and Murrell, 2005). Moreover, all MOB known so far except the acidophilic *Methylocella* (Dedysh et al., 2000) possess the *pmoA* gene, and its phylogeny corresponds largely to the 16S rRNA gene phylogeny (Kolb et al., 2003).

Due to a large number of sequences available from various habitats worldwide, the *pmoA* gene is an excellent proxy to study the correlation between MOB, environmental factors and geographical regions. Microbes are often perceived as opportunistic, fastgrowing organisms responding quickly to environmental changes. This may be a misconception: because microbes are excellent survivors when conditions become unfavourable, the imprint of past events may be preserved in contemporary communities. A variety of MOB are known to form cysts or exospores (Whittenbury et al., 1970), making them candidates for studying microbial biogeography.

We used a database-driven approach for analysing the *pmoA* genotypes found in Italian rice paddies compared with other rice-growing areas. We constructed clone libraries to expand our knowledge on *pmoA* diversity in the Italian paddy fields. We studied large-scale geographical patterns by compiling meta-information about geographical origins of paddies fields worldwide and combined it to the phylogenetic analysis of the respective *pmoA* sequences. On the smaller scale, we designed a factorial experiment to get deeper insights into the correlation of MOB with habitat and sites: within each of three Italian rice fields, 11–21 km apart, experimental plots were planted with the cultivars ROMA and KORAL. Fertilization and water management were similar in all three fields. This design aimed to distinguish between actual effects, e.g. the cultivar planted, and contingencies due to the different pre-experimental histories of the different fields, if any. The choice of cultivars was motivated by previous work showing both cultivars supporting MOB and $CH_4$ oxidation (Bosse and Frenzel, 1998; Eller et al., 2005).

The MOB populations are fully developed at late tillering/panicle initiation (Eller and Frenzel, 2001; Eller et al., 2005). Hence, we sampled at that growth stage rice roots and paddy soil. Because our focus was on population structures, we extracted DNA and used *pmoA* as a functional marker gene. Fingerprints from terminal restriction fragment analysis (T-RFLP) were used to explore the association between methanotrophic communities and cultivars, microhabitats, and/or fields by multivariate analyses.

## 2.3 Results

An existing *pmoA* nucleotide sequence database with approximately 600 sequences (Knief et al., 2006) was extended with publicly available and new sequences from this study ($n$ = 4000). It comprises *pmoA* sequences from various environments also covering part of the *amoA* diversity (about 6 % of the total database). The *amoA* gene (encoding the ammonia monooxygenase subunit A) is homologous to the *pmoA* gene and is often co-amplified with *pmoA* primers (Holmes et al., 1995). This database was the backbone for the following analyses. Phylogenetic clusters referred to below are defined and named according to cultured MOB or, for environmental clusters without cultured representatives, in relation to the nomenclature of microarray probes targeting the respective groups (Bodrossy et al., 2003; Stralis-Pavese et al., 2004; Vishwakarma et al., 2009).

*MOB in Italian wetland rice: comparative sequence analysis*

To get deeper insights into *pmoA* diversity in Vercelli rice fields, we combined 292 clone sequences obtained in this study with about 200 sequences from previous work (Henckel et al., 1999; 2000a; 2001; Horz et al., 2001; Shrestha et al., 2008). Phylogenetic analysis showed a wide distribution of Vercelli sequences throughout the entire *pmoA* diversity (Fig. 1). However, sequences belonging to upland soil clusters that are assumed to be responsible for the consumption of atmospheric methane, for example, USC- α, USC- γ (Knief et al., 2003) and the clusters JR-1, JR-2 and JR-3 (Horz et al., 2005), were not detected. One exception might be a tropical upland soil cluster in which one sequence from Vercelli is so far the only representative from a high-methane environment. This cluster together with the environmental clusters RA21, M84-P22 and M84-P105 are phylogenetically positioned between *pmoA* and *amoA*. Within type II MOB, the sequences from Vercelli showed a close relationship to *Methylocystis* while *Methylosinus*-related sequences were not detected. Furthermore, one sequence fell within the MO3 cluster consisting of sequences obtained from various low- and high-methane environments. Most Vercelli sequences affiliated to type Ib were related to *Methylocaldum,* or fell into an environmental cluster showing no close relationship to any cultivated MOB. This cluster is composed entirely of rice field sequences from various geographical origins. We therefore refer to it as RPC-1 (Rice Paddy Cluster 1; Fig. 1). Most of the sequences affiliated to type Ia grouped with

*Methylomonas*. Further sequences showed a close relationship to *Methylobacter* species or grouped within a second environmental cluster dominated by *pmoA* sequences from rice paddies (Rice Paddy Cluster 2, RPC-2; Fig. 1). Like RPC-1, these sequences originate from various geographical regions: Uruguay (Ferrando and Tarlera, 2009), China (Zheng et al., 2008) and Italy.

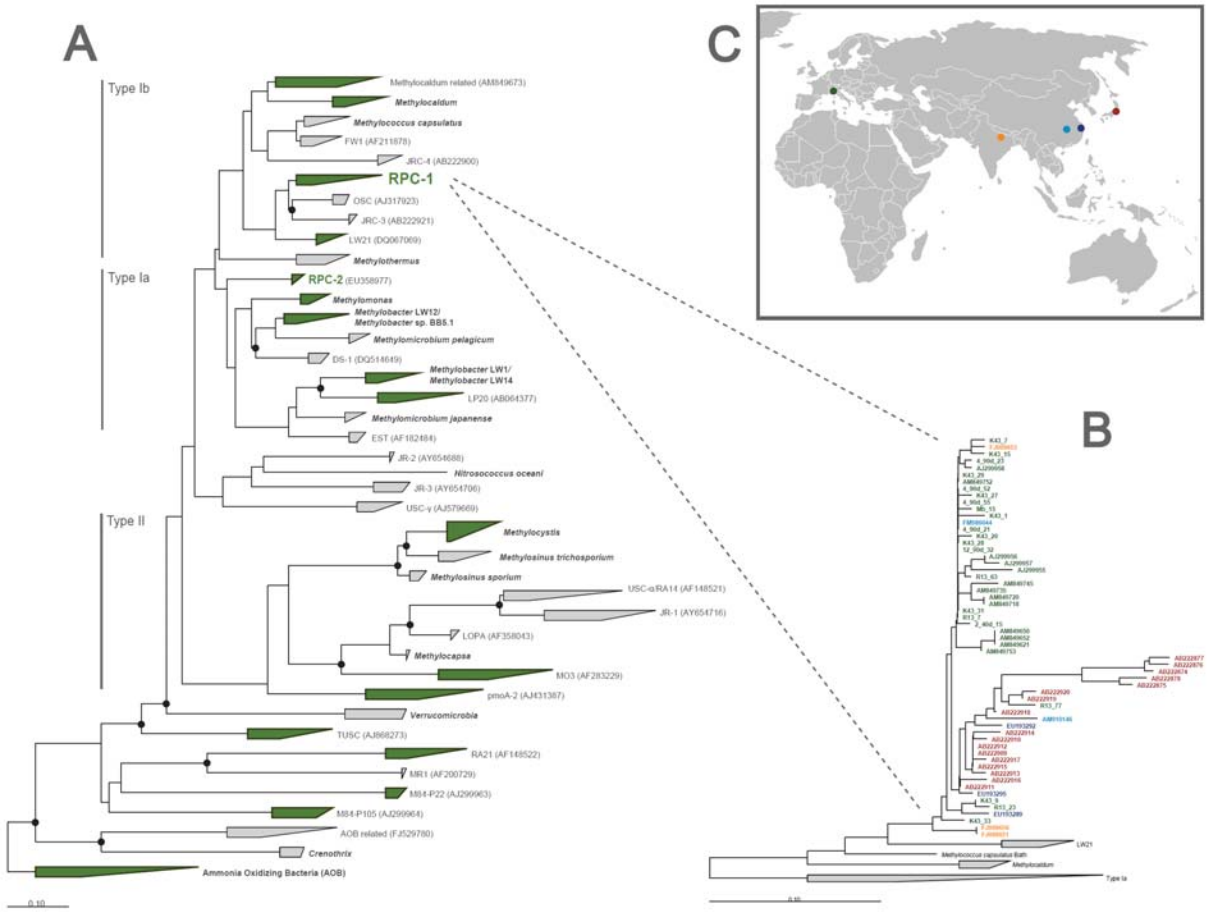


**Fig. 1.** Phylogenetic relationship of partial *pmoA* sequences based on deduced amino acid residues. Only sequences containing at least 140 amino acid positions were included in the analysis. A. Neighbor Joining tree showing the distribution of *pmoA* sequences retrieved from Vercelli rice paddies compared to public database sequences from various environments: Clusters containing Vercelli clone sequences are shown in green. Environmental clusters were named according to representative clones and/or in relation to the denotation of *pmoA* microarray probes. GenBank accession numbers of representative clones are given in brackets. Closed circles mark nodes that were verified by a Maximum likelihood tree. The scale bar represents 0.1 changes per amino acid position. B. Detailed view on the Rice Paddy Cluster 1 (RPC-1) which is composed entirely of rice paddy sequences from various geographical origins C. Geographical origins of the RPC-1 sequences.

*MOB in Italian wetland rice: fingerprint analysis*

The T-RFs were binned according to phylogenetic affiliations into a total of 10 operational taxonomical units (OTUs; Table 1). After binning and standardization, two to eight OTUs per individual sample were retained. Constrained correspondence analysis showed two clusters separating soil and root samples (Fig. 2). The OTUs affiliated to *Methylocaldum*, *Methylomonas* and *Methylobacter* (type I MOB) were almost exclusively detected in root samples, whereas soil samples were characterized by a higher abundance of type II MOB, the RA21 group and ammonia oxidizers. Soil samples from different locations were clearly separated, while different rice cultivars were not. This basic pattern was also preserved in non-metric multidimensional scaling (NMDS) analysis (Fig. S1).

**Table 1.** Affiliation of operational taxonomic units (OTUs) to phylogenetic groups of methanotrophs (Type I and Type II) or ammonium oxidizing bacteria (AOB). Binning was based on an *in-silico* analysis and cross-checked by T-RFLP analysis of clones. Clusters are defined in Fig. 1. [a] These sequences cluster between methanotroph *pmoA* sequences and *amoA* sequences from ammonium oxidizers and lack cultivated representatives.

| *OTU* | *Genus/Cluster* | *Subdevision of Proteobacteria* | *Type* |
|---|---|---|---|
| 46 | *Nitrosospira* | beta | AOB |
| 58 | RA21 group | | Others[a] |
| 79 | *Methylocaldum* related, RPC-1 | gamma | Ib |
| 114 | *Nitrosospira,* M84-P22 group, TUSC | beta | AOB Others[a] |
| 241,349, 505,531 | *Methylobacter / Methylomicrobium,* LP20 group, RPC-2 | gamma | Ia |
| 244 | *Methylocystis* | alpha | II |
| 437 | *Methylomonas* | gamma | Ia |

[a] These sequences cluster between methanotroph *pmoA* sequences and *amoA* sequences from ammonium oxidizers and lack cultivated representatives.

The hierarchy of factors explaining the variation of MOB communities was explored with a multivariate regression tree (MRT) (De'Ath, 2002). An MRT explains the variation of a multivariate response (the relative abundance of OTUs) using numeric or categorial explanatory variables (e.g. different locations or cultivars). The binary splits

are produced by minimizing the impurity within, and maximizing the heterogeneity between groups. The primary split separated soil and root samples (Fig. 3) consistent with the separation in ordination analysis (Fig. 2). This split is mainly characterized by the presence (on roots) or absence (in soil) of type I-related OTUs, and of *Nitrosospira* that was present in soil, but not on roots. Soils were further separated according to sites, while only the fourth-level split indicated a slight effect of cultivars on the methanotrophic community (Fig. 3). Hence, in spite of different algorithms, all analyses produced very similar results.

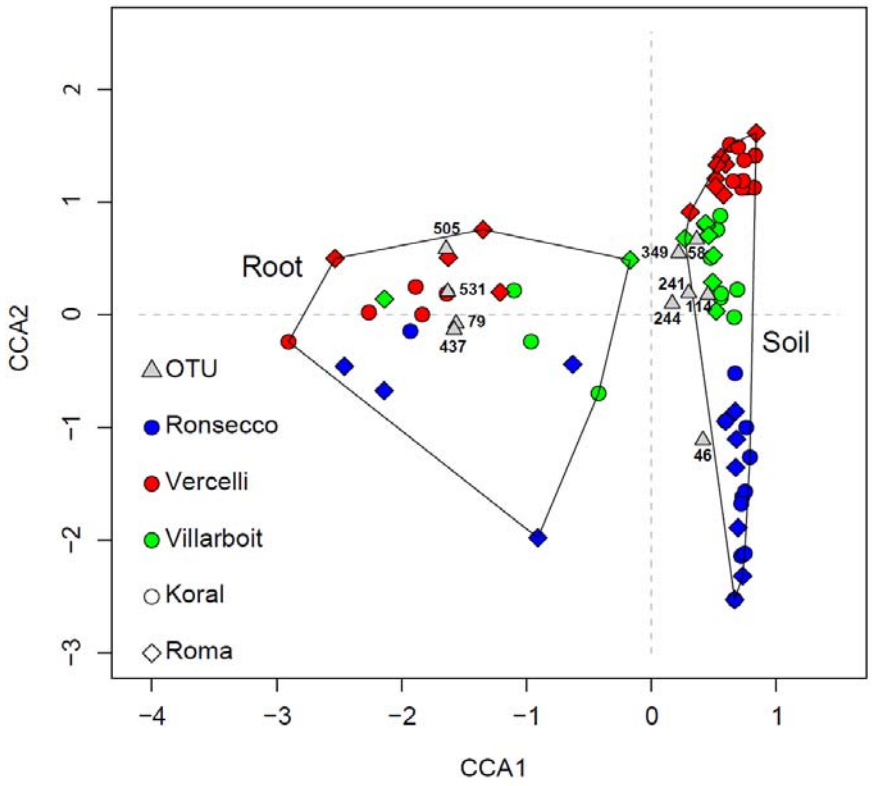


**Fig. 2.** Constrained correspondence analysis calculated from standardized T-RFLP data. Constraints used: micro habitat (roots versus soil), geographical location (Ronsecco versus Vercelli versus Villarboit), and rice variety (Roma versus Koral). Explained variance: 58 % of total inertia ($P$ = 0.005). OTUs are affiliated to MOB in Table 1.

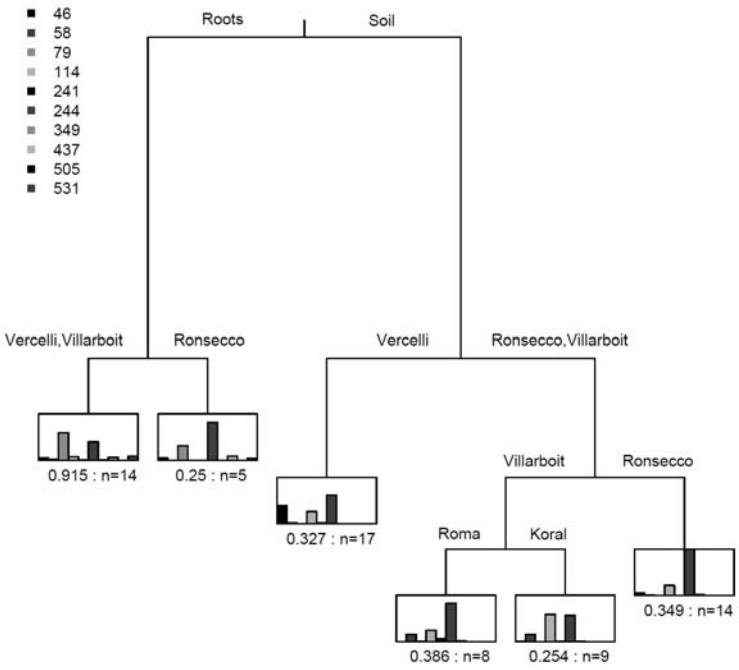


**Fig. 3.** MRT calculated from standardized T-RFLP data. The tree is based on the sums of squares in one group about the group mean. The bar plots show the multivariate species mean at each leaf and *n* depicts the numbers of cases at each leaf. OTUs are affiliated to MOB in Table 1.

## 2.4 Discussion

Previous studies on MOB diversity in specific environments are based on comparably small clone libraries. We combined data from several studies comprising about 500 *pmoA* sequences in total to obtain a more detailed view on MOB in Vercelli rice fields. The results indicate the presence of a highly diverse, but distinct community. They furthermore show a wide geographical distribution of *pmoA* genotypes that seem to be adapted to paddy fields. However, focusing on a smaller geographical scale, differences in the MOB communities were detected even at fields located only a few kilometers apart. Soil and root samples were characterized by different MOB communities, whereas the rice variety had no effect.

*MOB in wetland rice: biogeography at the global scale*

The MOB population in Vercelli rice fields shares some components that are common to wetland rice fields around the world. Within the type Ia MOB, a large amount of *pmoA* sequences retrieved from paddy fields show high identity (91–92 %) to *Methylomonas methanica*. Furthermore, several environmental *pmoA* clusters consist entirely of, or are dominated by rice field sequences. The RPC-1 represents the largest cluster comprising *pmoA* clones from rice fields distributed over all Eurasia (this study, Horz et al., 2001; Jia et al., 2007; Qiu et al., 2008; Shrestha et al., 2008; Zheng et al., 2008; P. Vishwakarma, unpublished) and might reflect the spread of *Oryza sativa* ssp. *japonica* from its native area in East Asia throughout the tropics and subtropics. RPC-2 is dominated by paddy field sequences from Italy, Asia and South America, whereas cluster JRC-3 consists of *pmoA* sequences retrieved so far only from Japanese and Chinese rice paddies (Jia et al., 2007; Qiu et al., 2008; Zheng et al., 2008). For the JRC-4 comprising sequences from Asia and South America, an isolate could be obtained very recently from Uruguayan rice fields (Ferrando and Tarlera, 2009). It showed congruent phylogeny of *pmoA* and 16S rRNA with *Methylococcus capsulatus* and *Methylocaldum szegediense* as closest cultivated relatives (92 % and 91 % 16S rRNA gene identity, respectively) and might be the first representative of a new MOB genus. A lot of sequences from nearly all studied paddy field sites cluster near *Methylocaldum*. All of these rice clusters, apart from the RPC-2, could be affiliated to type Ib MOB based on *pmoA* phylogeny. RPC-2 shows an ambiguous relation to either type Ia or type Ib, depending on the method used for tree construction.

All rice clusters combine sequences from at least three geographically separated paddy fields suggesting a certain adaptation to the rice field environment. The studies are based on clone libraries of very different sizes ranging from 30 to 40 clones (Jia et al., 2007; Zheng et al., 2008; Vishwakarma et al., 2009; Ferrando and Tarlera, 2009) to approximately 500 in this work. Even for the Italian clone library, rarefaction curves did not level off (Fig. 4), indicating a still incomplete picture of total MOB diversity and a considerable contribution of the 'rare biosphere'. Hence, an increasing sampling effort might detect some clusters in other geographical regions or in environments other than paddy fields. However, in-depth studies using the recently developed high-resolution *pmoA* microarray (Bodrossy et al., 2003; Stralis-Pavese et al., 2004) did not detect these clusters in peat, landfill and gleyic soil (Cebron et al., 2007; Chen et al., 2008; Héry et al., 2008), whereas they were detected in an Indian rice paddy (Vishwakarma et al., 2009). These studies strongly support our view of a rice-specific methanotrophic community.

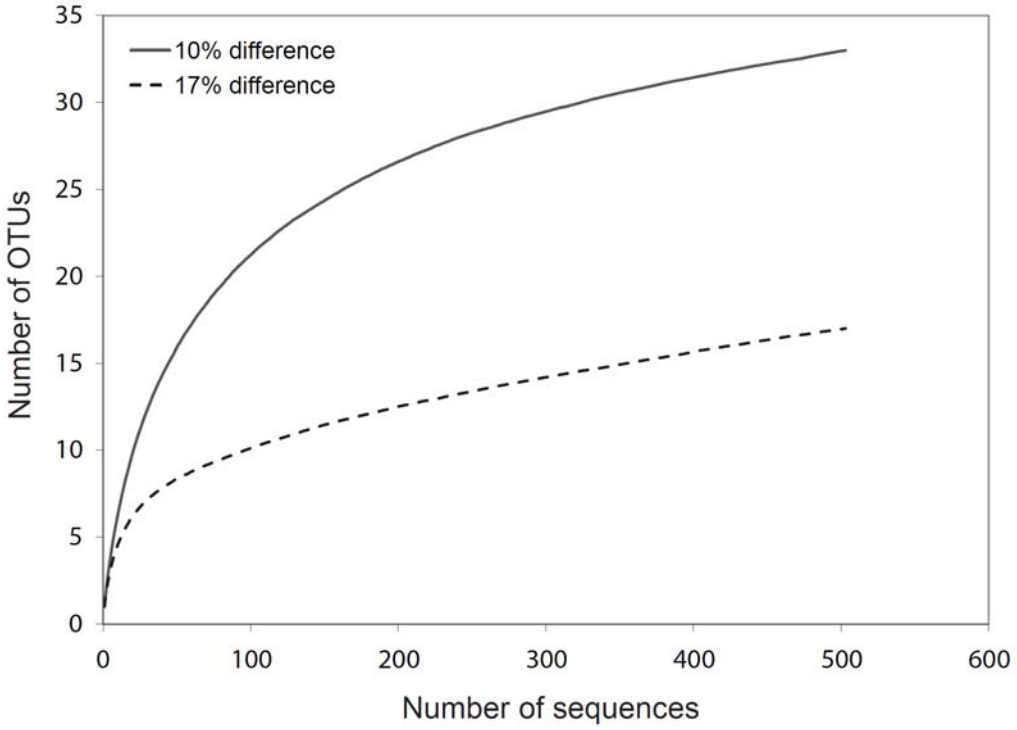


**Fig. 4.** Rarefaction analysis of collected *pmoA*/*amoA* sequences from Vercelli rice fields. The analysis is based on sequences retrieved with both primer pairs A189f/mb661 and A189f/A682r). Nucleotide sequences were grouped as OTUs using the distance levels 10 % and 17 %. These *pmoA* distances correspond to the 3 % and 5 % 16S rRNA distance assuming a 3.5 times higher nucleotide substitution rate (Heyer *et al.*, 2002).

The primer choice has a large impact on the detectable diversity. A189f/A682r (Holmes et al., 1995) and A189f/mb661r (Costello and Lidstrom, 1999) are the predominant primers used for studying *pmoA* diversity. Whereas A682r was widely used in earlier studies (Reay et al., 2001; Kalyuzhnaya et al., 2002; Radajewski et al., 2002), more recent publications show a preference for mb661r (Chen et al., 2008; Zheng et al., 2008; Ferrando and Tarlera, 2009). One reason for this change is the discrimination of mb661r against the homologous *amoA* of ammonia oxidizers. However, mb661r also discriminates against the clusters USC- α and RA21 (Bourne et al., 2001). For the Vercelli sequence pool, several other clusters located in between the MOB and ammonium oxidizing bacteria (tropical upland soil cluster, M84-P22 and M84-P105) have only been retrieved using A682r (Table S1). However, clusters within the type Ia methanotrophs, such as the RPC-2 and *Methylobacter*-related genotypes, seem to be preferentially amplified by the mb661r primer. Therefore, the combined use of both primer sets will reveal the most complete picture of methanotrophic diversity (McDonald et al., 2008).

That no sequences belonging to any of the upland soil clusters were obtained might seem trivial, but crop rotation between wetland rice and other cereals is quite common, and the Italian fields lay fallow during winter. Hence, rice field MOB may at least temporarily be exposed to low methane concentrations. Indeed, re-analysing the phylogenetic position of sequences retrieved from a Chinese paddy field (Zheng et al., 2008) revealed two sequences grouping within USC- γ.

*MOB in Italian wetland rice: biogeography at the regional scale*

Besides considering the large geographical scale, we compared the MOB communities in three very closely located paddy fields within Northern Italy. This comparison was carried out by T-RFLP analysis resulting in similar T-RFs found in all soils. However, in spite of a nearly identical agricultural treatment, the three sites showed significantly different community patterns based on the relative abundance of specific T-RFs (Fig. 2 and 3). The reason for these differences might be found related to the history of the soils. A variety of MOB are able to form cysts or exospores (Whittenbury et al., 1970; Bowman et al., 1993) and are believed to remain viable for more than a century (Rothfuss et al., 1997). Rice soils contain a large yet inactive population of MOB acting as a seed bank (Eller and Frenzel, 2001; Eller et al., 2005). From this seed bank, past events might have activated different sub-populations from time to time. These events

could be of different nature and we can only speculate about it. Different agricultural practices in the past might be of importance as well as natural variability at the three sites. A combination of all effects might have resulted in different community compositions at each site that were conserved in the seed bank. Changes in this seed bank may be slow, but once established, they might persist for quite a time providing a complex community from which the actual environment selects a subset.

*MOB at the field scale: influence of habitat and rice variety*

The T-RFLP profiles did not indicate an effect of the rice varieties on MOB communities. However, both cultivars studied belong to *O. sativa* ssp. *japonica.* The Italian germplasm in particular has recently been found to be genetically quite homogeneous (Lupotto, unpublished). Analysing more distantly related cultivars may give further insights into the potential role of rice varieties shaping MOB communities.

Ordination methods as well as multivariate regression analysis all resulted in a clear separation of soil and root samples. Growth proliferation of type I MOB on rice roots has been reported previously (Bodelier et al., 2000b; Eller and Frenzel, 2001; Horz et al., 2001; Shrestha et al., 2008). The rhizosphere is a dynamic environment providing highly variable concentrations of oxygen and methane (Gilbert and Frenzel, 1998). In soil microcosms, type I methanotrophs respond most rapidly to different $O_2/CH_4$ ratios, whereas type II are apparently less responsive becoming active only with time (Henckel et al., 2000b). Considering the transient nature of rhizosphere oxygenation (Flessa and Fischer, 1992), type I may be preadapted to this ephemeral microenvironment.

*Conclusions*

At the large geographical scale, a number of environmental clusters could be observed that were predominantly found in paddy fields around the world. Remarkably, nearly all clusters could be assigned to type Ib MOB. This suggests the existence of MOB diversity patterns not only for low-methane environments – characterized by the predominance of upland soil clusters – but also for a high-methane environment.

At the small geographical scale, we found different MOB community compositions at three closely located sites. Considering the ability of many MOB to survive adverse conditions for decennia, we suggest that historical contingencies are of major importance shaping a particular population (Martiny et al., 2006; Ge et al., 2008).

With the current agronomical practice being nearly identical, these forces may act even at the scale of some kilometres. The concept of a microbial seed bank describes best the coexistence of an active population on rice roots with a large soil-borne reservoir of viable yet inactive cells (Eller et al., 2005; Pedros-Alio, 2006; Lliros et al., 2008). This case suggests that changing conditions may select in the future other MOB from the seed bank. Nevertheless, each paddy field stays unique holding its specific and characteristic MOB fingerprint.

## 2.5 Experimental procedure

*Sampling site*

The samples were collected from rice fields situated on alluvial soils in the lowlands of the rivers Po and Sesia (Vercelli, Italy). Three locations, located 11–21 km apart and characterized by similar soil textures (Table 2), were chosen.

**Table 2.** Location and soil parameters of sampling sites.

| Location | Geographic coordinates | Texture [%] | | | Soil Analyses | | |
|---|---|---|---|---|---|---|---|
| | | Sand | Silt | Clay | Organic Carbon | C/N | pH |
| Vercelli | 08°22'25.89"E 45°19'26.98"N | 67 | 30 | 3 | 2.5 | 9.0 | 6.2 |
| Ronsecco | 08°15'16.13"E 45°16'37.48"N | 61 | 36 | 3 | 2.4 | 9.6 | 5.9 |
| Villarboit | 08°19'19.94"E 45°27'27.07"N | 61 | 35 | 4 | 2.6 | 13.1 | 5.7 |

*Sampling procedure*

All fields have been planted to wetland rice since the beginning of last century at least. It was sampled in July 2006. In that year, fields were flooded in April with rice being sowed at end of April/beginning of May. In each field, two plots were laid out planted to cultivars Roma and Koral respectively. Plot width was 1.2–1.5 m, while length varied from 8 to 35 m. The MOB in Italian rice fields have no spatial structure (Krause et al., 2009). Hence, a simple transect sampling was used taking core and root samples along the main axis of the plots with a sample-to-sample distance of 1–3 m. Depending on the size of the plot, four to five samples were taken. Soil was sampled with a corer (inner diameter 6.5 cm) down to a depth of 10 cm and divided into two layers equally deep, the upper and lower representing the rooted and the bulk soil respectively. Soil was transferred into plastic bags, kneaded to homogenize, and sub-sampled (1.5 ml). Roots from two rice plants were cut with scissors into pieces 2 cm long, mixed and packed in plastic bags. Samples were frozen on site with dry ice. Before handling the next samples, all instruments were cleaned with ethanol. Samples were kept on dry ice during transport and stored at -20°C later on till processing.

*DNA extraction from root samples*

Total DNA from roots was isolated using the DNAeasy Plant Maxi Kit (Qiagen). It followed the manufacturer's instructions for manual plant tissue disruption under liquid nitrogen. DNA was purified afterwards using the Wizard DNA Clean Up System (Promega) according to the manufacturer's instructions.

*DNA extraction from soil samples*

DNA isolation from soil samples was performed as previously described by Stralis-Pavese et al. (2004). Briefly, 0.3 g soil was re-suspended in sodium phosphate buffer (pH 7.0) supplemented with CTAB and lysozyme. Cells were disrupted by beat beating followed by proteinase K treatment. DNA was further purified by phenol-chlorophorm-isoamyl alcohol and chlorophormisoamyl alcohol extraction. Potassium acetate was added for humic acids precipitation. DNA was bound to a silica matrix (FastDNA spin kit for soil, QBiogene) and washed with ethanol. Elution of DNA was performed in EB buffer (Qiagen).

*Cloning, sequencing and phylogenetic analysis of pmoA genes*

*pmoA* clone libraries were generated from field samples (soil and roots from different locations) and amended by clones retrieved from greenhouse experiments including rice field soil from Vercelli. In total, 292 clones were randomly selected for comparative sequence analysis. *pmoA* genes were amplified using the forward primer A189f (5′-GGN GAC TGG GAC TTC TGG) and the reverse primers A682r (5′-GAA SGC NGA GAA GAA SGC) (Holmes et al., 1995) or mb661 (5′-CCG GMG CAA CGT CYT TAC C) (Costello and Lidstrom, 1999). Three reactions of 50 ml were carried out per sample. 100 ng template DNA was mixed with 2.5 U of Taq Polymerase (Invitrogen), 66 pmol of each primer, 0.02 mg bovine serum albumine (Roche), 5 % (v/v) DMSO, 25 ml Masteramp 2x PCR Premix F (Epicentre Biotechnologies) and filled up with molecular grade water (Sigma). The touchdown PCR was carried out with an initial denaturing step at 94°C for 5 min, followed by 11 cycles of 1 min at 94°C, 1 min at 62°C (touchdown 1°C per cycle) and 1 min at 72°C. Further 24 cycles were carried out for 1 min at 94°C, 1 min at 52°C and 1 min at 72°C. Final elongation was performed for 10 min at 72°C. PCR products were analysed by 1 % agarose gel electrophoresis and visualized by staining with ethidium bromide. PCR products of parallel samples were pooled and purified using the GenElute PCR clean-up Kit by Sigma. Purified PCR

products were cloned into the vector pGEM-T (Promega) and transferred into competent cells of *E. coli* JM109. Transformants were selected by blue-white screening and analysed by colony PCR using the primers T7 (5′-TAA TAC GAC TCA CTA TAG GG) and M13rev_29 (5′-CAGGAA ACA GCT ATG ACC) (MWG Biotech).

The PCR products of the appropriate size were sequenced at ADIS, MPI for Plant Breeding Research (Cologne, Germany). Sequencing was carried out in both directions. Sequences were assembled and vector sequence was deleted using the SeqMan software (DNA-Star software package, Lasergene). Sequences were compared with the GenBank database using the NCBI BLAST. Phylogenetic tree construction was based on 140 deduced amino acid residues. The analysis was performed using the neighbor joining method implemented in the ARB software package (Ludwig et al., 2004). The overall tree topology was compared with a tree calculated using maximum likelihood and nodes verified by both methods were marked.

The *pmoA* and *amoA* sequences obtained in this study were deposited at the EMBL nucleotide sequence database under the accession numbers FN599861-FN600113, and FN600122-FN600155.

*T-RFLP analysis of pmoA genes*

*pmoA* genes were amplified using the FAM-labelled forward primer A189f_FAM and the reverse primer A682r as described for the clone library. 100 ng PCR product was digested by mixing with 10 U of MspI enzyme (Fermentas) and 1 ml Tango buffer + BSA (Fermentas) filled up to 10 ml with molecular grade water (Sigma) and incubated at 37°C for 3 h. The enzyme was inactivated at 65°C for 20 min. The following purification was performed using the AutoSeq G-50 columns (Amersham Biosciences). One microlitre of each purified sample was mixed with 0.2 ml of DNA fragment length standard (MapMarker 1000; Bioventures) and 11 ml Hi-Di Formamid (Applied Biosystems). The samples were denatured for 3 min at 94°C and T-RFLP analysis was carried out using the GeneScan ABIPrism 310 (Applied Biosystems). Analysis of the T-RF patterns was carried out using the appropriate analysis software (GeneScan Analysis Version 2.1, Applied Biosystems).

*Statistical analysis of T-RFLP profiles*

Statistical analysis was performed using the R software environment for statistical computing and graphics (version 2.8.1) (R Development Core Team, 2008). The T-RFs

were binned to OTUs based on an *in silico* analysis of *ca*. 500 sequences from field and greenhouse experiments with soil from Vercelli. After binning, a data set was generated consisting of T-RF sizes in bp and peak heights in fluorescence units for each sample. The T-RF profiles were quality-checked as described before (Krause et al., 2009) and standardized (Dunbar et al., 2000).

The NMDS and constrained correspondence analysis was performed using *metaMDS* and *cca* provided by the *vegan* package (version 1.15-1) (Oksanen, 2008). In NMDS analysis, the Bray–Curtis distance was chosen for creating the dissimilarity matrix. The MRT was constructed using the *mvpart* package (version 1.2-6) (De'Ath, 2007). Rarefaction curves were computed using DOTUR (version 1.3) and the furthest neighbour algorithm (Schloss and Handelsman, 2005).

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## 2.7 Supplementary information

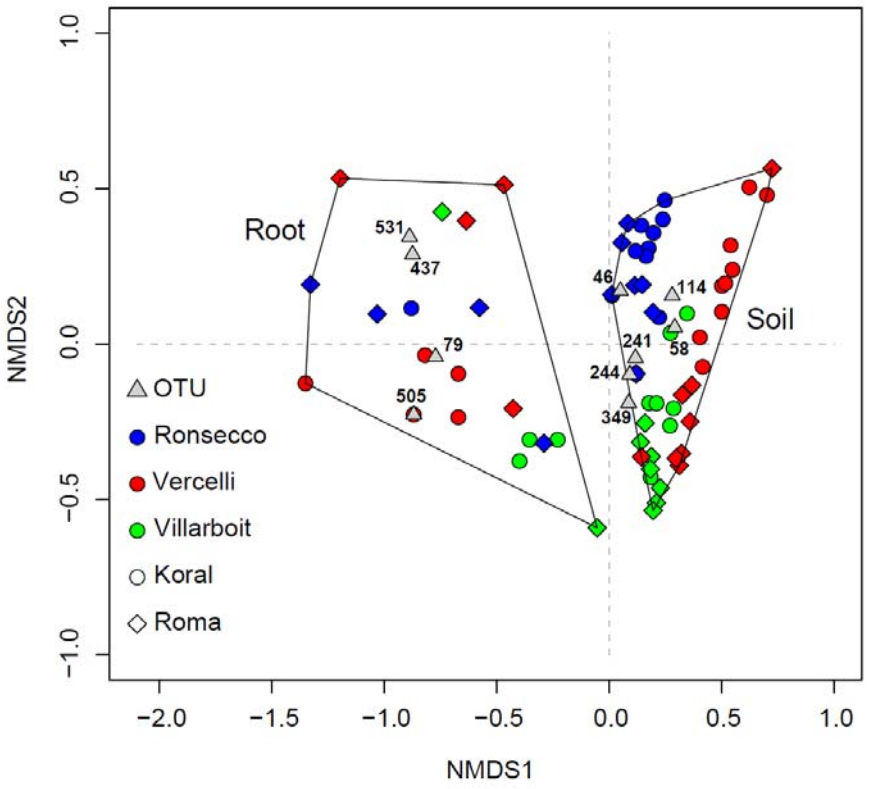


**Fig. S1.** NMDS plot calculated from standardized T-RFLP data. The calculation is based on the Bray-Curtis distance (Plot stress = 0.14).

**Table S1.** Phylogenetic distribution of *pmoA/amoA* sequences from Vercelli rice fields detected by the different reverse primers A682r and mb661. Clusters are defined in Fig. 1.

| **Genus/Cluster** | **Type** | **Reverse primer** | |
|---|---|---|---|
| | | mb661 | A682r |
| *Methylomonas* | Ia | 55 | 13 |
| *Methylobacter* LW12/BB5.1 | Ia | 11 | 0 |
| *Methylobacter* LW1/LW14 | Ia | 22 | 0 |
| LP20 | Ia | 0 | 4 |
| RPC-2 | Ia | 1 | 0 |
| *Methylocaldum* | Ib | 4 | 1 |
| *Methylocaldum* related | Ib | 36 | 22 |
| RPC-1 | Ib | 10 | 24 |
| LW21 | Ib | 1 | 0 |
| *Methylocystis* | II | 93 | 151 |
| MO3 | II | 0 | 1 |
| pmoA-2 | II | 0 | 1 |
| TUSC | Others* | 0 | 1 |
| RA21 | Others* | 0 | 6 |
| M84-P22 | Others* | 0 | 2 |
| M84-P105 | Others* | 0 | 2 |
| Ammonia oxidizers | AOB | 0 | 42 |
| Total | | 233 | 270 |

*These sequences cluster between methanotroph *pmoA* sequences and *amoA* sequences from ammonium oxidizers and lack cultivated representatives.

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# 3. Spatial heterogeneity of methanotrophs a geo-statistical analysis of *pmoA*-based TRFLP patterns in a paddy soil

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## 3.1 Abstract

Despite numerous studies on methanotrophs, virtually nothing is known about their spatial heterogeneity in nature. These patterns, however, have strong influences on the interpretations made from analysing microbial processes and community structure. Here we report the first use of geostatistics to analyse the spatial heterogeneity of methanotrophs in a rice field soil (Vercelli, Italy). We used the gene encoding the particulate methane monooxygenase, *pmoA*, for terminal restriction fragment length polymorphism (T-RFLP) analysis. The profiles obtained were compared using a pseudo-variogram analysis to study autocorrelation as a function of distance. We demonstrated that there was no large-scale spatial structure at this study site, but a micro-scale spatial structure could not be excluded. A species accumulation curve with all terminal restriction fragments revealed that even 75 samples were insufficient to cover the diversity of methanotrophs in a rice field. However, a species accumulation curve of methanotrophs defined as operational taxonomic units validated from a clone library with 90 % coverage demonstrated saturation after approximately 15 samples. The results of this study have consequences for studying the diversity and function of methanotrophs. In this agroecosystem population structure showed no spatial pattern implying that both a systematic and random sampling design would be adequate.

## 3.2 Introduction

Methanotrophs are a physiologically unique group of bacteria that utilize methane as sole carbon and energy source. They belong to the *Gammaproteobacteria* (type I methanotrophs) and *Alphaproteobacteria* (type II methanotrophs). Both groups oxidize methane via methanol and formaldehyde to carbon dioxide (Hanson and Hanson, 1996; Bowman, 2006; McDonald *et al.*, 2008; Trotsenko and Murrell, 2008). A key enzyme in this pathway is the particulate methane monooxygenase (pMMO), which is present in all known methanotrophs except the acidophilic *Methylocella* spp. (Theisen *et al.*, 2005). Hence, the *pmoA* gene, which encodes the α subunit, can be used as a molecular marker for the identification of methanotrophs in environmental samples (McDonald and Murrell, 1997).

In wetland rice fields, methanotrophs can reduce the potential methane emissions up to 80 % (Conrad and Rothfuss, 1991) and thus play an important role in the global methane budget. Consequently, the physiology, diversity and ecology of methanotrophs have been studied in detail (Hanson and Hanson, 1996; Conrad, 2007; McDonald *et al.*, 2008). However, information on their spatial heterogeneity in nature is lacking and has been rather neglected when studying methanotrophs.

The distribution of microorganisms in the environment is heterogeneous (Franklin and Mills, 2003). Bacterial communities are structured not only by the physiology and ecological properties of the members, but also by environmental parameters. These gradients have to be evaluated when designing field studies of bacterial diversity and function.

A powerful tool for gaining insight into the spatial structure is geostatistics. Geostatistics originate from soil science and are widely used for quantifying spatial patterns (Legendre and Legendre, 1998; Ettema and Wardle, 2002). It is based on the assumption that spatial variability is autocorrelated, i.e. locations close to each other are more similar than those further apart. A method to identify the spatial structures is variogram analysis. Generally, semi-variances between samples are calculated and plotted against their spatial separation; the slope indicates whether a spatial structure is present (Ettema and Wardle, 2002).

In microbial ecology, so far, only a few studies have used geostatistics, e.g. Franklin and colleagues (2002), Mummey and Stahl (2003), Nicol and colleagues (2003), Ritz and colleagues (2004) and Bengtson and colleagues (2007). For example,

the use of geostatistics has shown that the spatial structure of microbial communities in agricultural soils can greatly differ from site to site. Grundmann and Debouzie (2000) demonstrated with a pure culture experiment of ammonia and nitrite oxidizers using an agricultural soil cultivated with maize that they aggregate at a millimetre scale. In a field study, Franklin and Mills (2003) applied amplified fragment length polymorphism (AFLP) to the total bacterial community and pointed out that the bacterial distribution can be highly structured over a distance of 30 cm to more than 6 m in a wheat field. In sharp contrast to both of these studies, Robertson and colleagues (1997) observed no spatial variability in the culturable bacterial population in a monospecific crop field, even though soil properties varied. Hence, the possible spatial variability has to be evaluated when microbial processes are analysed (Robertson *et al.*, 1997).

Our study was performed to address the general need for more information about the spatial heterogeneity of bacterial populations in agricultural systems. We used methanotrophs from a rice field as a model system. We measured *pmoA*-based terminal restriction fragment length polymorphism (T-RFLP) and applied geostatistics to provide insights in the spatial structure. Moreover, this work could be applied to develop an optimal sampling strategy for diversity studies in rice fields.

## 3.3 Results and discussion

*Spatial structure*

The applied sampling scheme ensured that a representative data set was recorded (Fig. 1). Our geostatistical analyses resulted in a pseudo-variogram depicting the spatial organization of methanotrophs (Fig. 2). The Jaccard dissimilarities of *pmoA*-based T-RFLP patterns averaged 0.64. There were no trends towards a change in dissimilarities with increasing separation distance, which implied no spatial structure at the study site sampled.

Considering the history of the study site, we can postulate a possible explanation for this result. The site has been planted with rice for more than 100 years (E. Lupotto, pers. comm.). Plowing and puddling of the flooded soil has effectively homogenized the topsoil, and when flooded this soil lacks horizontal gradients, i.e. the soil properties are fairly constant. If the plant root system and rhizosphere affect the distribution of microorganisms, as suggested by a study in a grassland (Mummey and Stahl, 2003), these effects would be even more similar throughout the site in the monospecific rice culture. Hence, the methanotrophic community would not be spatially structured. A second factor may be similarly important: by volume, the largest fraction of the flooded soil is anoxic enabling aerobic methane oxidation only at the soil surface layer and in the rhizosphere. Methanotrophs can form drought-resistant cysts and exospores (Whittenbury *et al.*, 1970; Bowman *et al.*, 1993) that are assumed to make up the largest fraction of the population (Eller and Frenzel, 2001). Methanotrophs have been reported to survive unfavourable conditions for up to 170 years (Rothfuss *et al.*, 1997). Hence, this long persistence in the environment will level out actual population changes, particularly if not the active but the total population is analysed.

In contrast, the medians of the boxes in the pseudo-variogram were significantly different at small distance classes (2.7–5.4 m, Fig. 2) and at large distance classes (18.9–32.5 m, Fig. 2). No significant differences for intermediate distance classes were observed. Differences at short distances pointed towards a slight spatial structure (Fig. 2), which is also underlined by the results of a local regression analysis (Fig. S1). Previous work has shown that on the micro-scale, e.g. comparing roots to the surrounding soil, the population structures can differ (Eller and Frenzel, 2001). However, an additional pseudo-variogram analysis on a small scale (< 10 m) did not reveal any spatial structure (data not shown). Hence, we assume that the scales at which

spatial heterogeneity might occur are mainly smaller than those measured. We cannot exclude a spatial structure at the millimetre scale, as shown for instance by Grundmann and Debouzie (2000).

*Species (operational taxonomic unit) coverage*

The assignment is based on a *pmoA* database containing more than 4000 sequences. From these sequences, 500 were obtained from Vercelli rice fields and a rarefaction analysis, an estimation of the total diversity based on a subsample, indicated a good coverage of methanotrophic diversity in this agroecosystem (data not shown). Terminal restriction fragments (TRFs) could be affiliated to the following operational taxonomic units (OTUs): *pmoA*/*amoA* like RA21 cluster (58 bp) *Methylococcus/Methylocaldum* (80 bp), *Methylosinus trichosporium* OB3B (146 bp), *Methylosinus/Methylocystis* (245 bp), *Methylomicrobium album* (350 bp). In addition, the 47 and 113 bp TRFs were affiliated to the *amoA* gene, which is partially covered with the applied primers. This assignment is consistent with previous work (Holmes *et al.*, 1999; Horz *et al.*, 2001; Reay *et al.*, 2001; Shrestha *et al.*, 2008). With this information, an additional pseudo-variogram analysis was performed using only the assigned OTUs. The pseudo-variogram was the same as that shown in Fig. 2, with a mean Jaccard dissimilarity of 0.59. Although there was no obvious spatial structure, we identified some indications of species interactions between type I and type II methanotrophs (Table S1). Henckel and colleagues (2000) found that type II methanotrophs are usually present or most active when environmental conditions in a rice field have become fairly constant, whereas type I methanotrophs are more active when environmental conditions are more variable, e.g. during drainage or flooding. They suggested that generally type I and II methanotrophs occupy different niches and coexist. Additionally, the impact of protistan grazing might have an effect on the distribution and abundance of methanotrophs, as shown by Murase and colleagues (2006).

*Species accumulation curve*

The results of our study have general implications for the study of diversity and function of methanotrophs. Demonstrating that a small sample size can representatively cover the *pmoA*-based TRFs in a rice field would simplify sampling effort and save costs and time. In our first analysis, all fragments were included and the curve did not flatten, which indicated that 75 samples are insufficient to cover all TRFs (data not

shown). Along these lines, Schloss and Handelsman (2004) illustrated that even approximately 56 000 partial 16S rRNA gene sequences did not cover the microbial census and are far from complete, as shown by a sharp slope of the rarefaction curve, which is similar in application to the species accumulation curve. Hence, we limited the analysis to the previously assigned OTUs. This species accumulation curve flattened, i.e. indicated that the curve was saturated at a sample size of approximately 60 (Fig. S2), and a manageable sampling unit of approximately 15 was found when a limit was set at 90 % OTU coverage. We believe that it is currently not feasible to cover an entire ecosystem, even for just a single functional group like the methanotrophs. Hence, the focus should be more on different community patterns, or on species and their ecological relevance than on covering every single species in an ecosystem. There are consequences for the sampling effort. In ecosystems with gradients the sampling strategy is of major importance, because all gradients have to be considered and a high number of samples are required. However, in systems, without gradients the samplings strategy, e.g. a systematic or random design, seems to be irrelevant and a small sample size can representatively cover the study site.

In summary, our results demonstrate that the methanotrophic community in the rice field studied had no obvious spatial structure. A structure on the millimetre scale is possible, but this still needs to be investigated. We were able to limit the sample size without losing important fragments. Although no spatial structure was found at the level of T-RFLP patterns, we demonstrated that there is a noteworthy difference in the occurrence of distinct methanotrophic phylogenetic groups. The pseudo-variogram analysis in connection with T-RFLP analysis is a suitable method for the identification of spatial structures. Hence, this method can be adapted to every kind of environment. Since this study is based on DNA, i.e. on the presence of gene copies, the results do not necessarily reflect the active community. Future analyses should concentrate on active methanotrophs since remarkable differences in the community structure based on the activity of single species are expected.

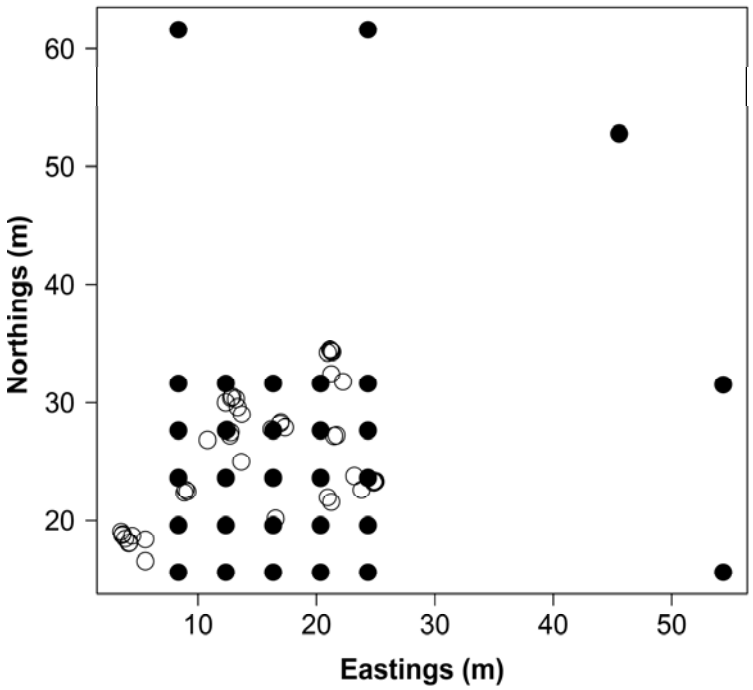


**Fig. 1:** Sampling design, illustrating the location of the sampling points. Filled circles represent grid cells and samples spaced further apart; open circles show samples of the random-walk transects. Soil samples were collected from a paddy rice field of the C.R.A. Unità di Ricerca per la Risicoltura (Vercelli, Italy) in autumn 2006 after drainage and harvest. A 60 x 60 m area of a rice field was sampled. In this area, 25 points were marked with 4 m between each point, forming a regular grid. Five points were chosen at random as the starting points of independent random-walk transects, as described elsewhere (Ritz *et al.*, 2004). In addition, five samples, 30 m apart, were taken. In total, 75 samples were collected. Each sample consisted of a 64 mm core taken from the rice field surface to a depth of approximately 6 cm.

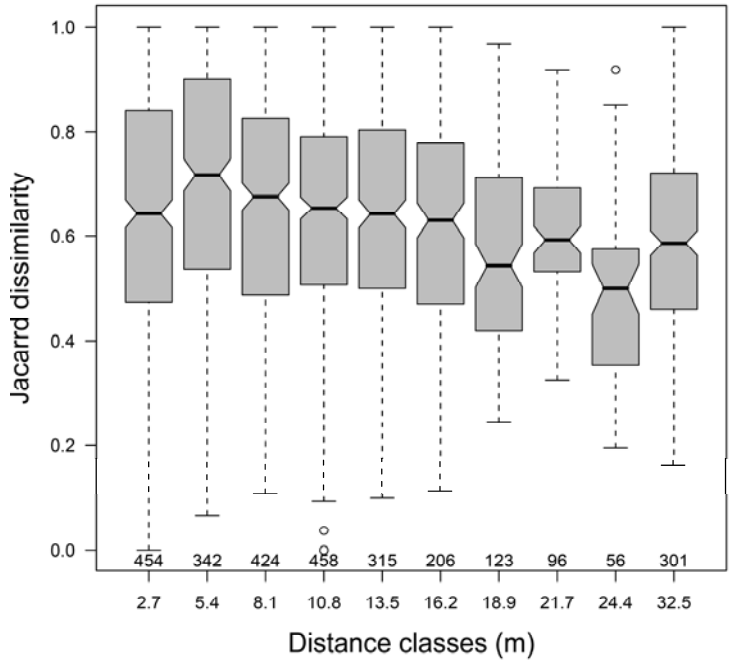


**Fig. 2:** Spatial heterogeneity, shown as a pseudo-variogram. Since T-RFLP analysis generates multivariate data, we followed the approach of Franklin and colleagues (2002) using the Jaccard coefficient as a measure of dissimilarity. Dissimilarities are shown as a series of box and whisker plots binned to distance classes. The horizontal lines in the boxes indicate the median. The bottom and top of each box indicate the 25 and 75 percentiles respectively. Whiskers are 1.5 times the interquartile range of the data, and points outside this range are classified as outliers. Notches are shown around each median. If the notches do not overlap, the medians are roughly significantly different at about a 95 % confidence level (McGill *et al.*, 1978). The numbers above the distance classes indicate the sample size. DNA was extracted following the protocol of Stralis-Pavese and colleagues (2004). Amplification of the *pmoA* gene was carried out in triplicates per sample and pooled afterwards followed by a *pmoA*-based T-RFLP analysis (Horz *et al.*, 2001). T-RFLP data were standardized using the relative abundances of TRF peak heights (Lüdemann *et al.*, 2000).

## 3.4 Acknowledgements

We thank Elisabetta Lupotto and the C.R.A. Unità di Ricerca per la Risicoltura for support at the rice fields in Vercelli, Italy. We thank Belinda Schneider and Alexandra Hahn for technical assistance. The study was financially supported by a grant from the Deutsche Forschungsgemeinschaft (ESF and EuroDIVERSITY-METHECO).

## 3.5 Supplementary material

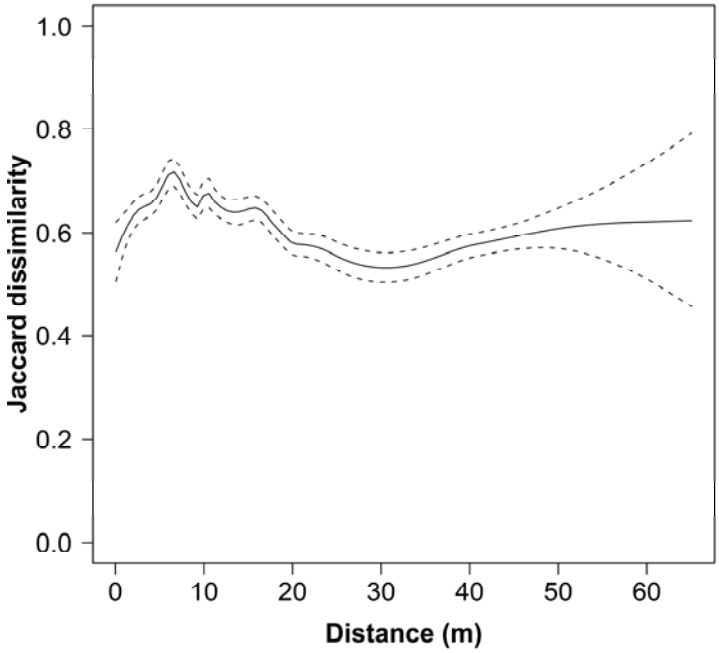


**Fig. S1.** Local regression fit of Jaccard dissimilarities plotted against spatial distance. The nearest neighbour bandwidth (a = 0.3; proportion of data used in each fit) was used. Dashed lines represent approximate 95% point-wise confidence intervals for the mean. Local regression was performed using the local regression software LOCFIT as implemented in the statistical software R (Loader, 1999; R Development Core Team, 2008).

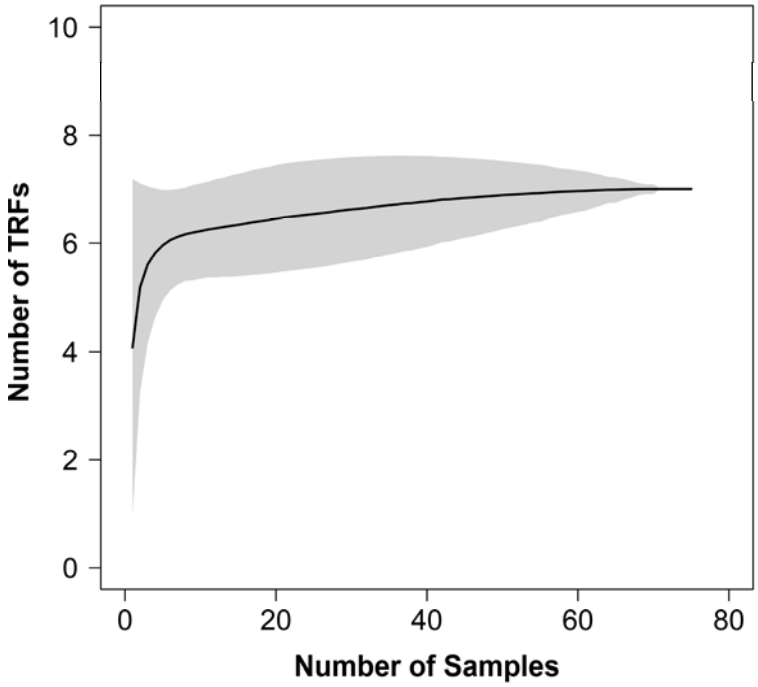


**Fig. S2.** Species accumulation curves of all OTUs using random accumulation of sites and a 95% confidence interval (shaded area). A randomization approach was used where the average species richness is calculated for a series of randomly pooled sites (Kindt and Coe, 2005). Each TRF was considered as an operational taxonomic unit (OTU).

**Table S1.** Spearman's rank correlation coefficients between TRFs using relative abundances; TRF affiliation: 47 bp, *Nitrosomonas/Nitrosospira*; 80 bp, *Methylococcus/Methylocaldum*; 113 bp, *Nitrosospira*; 245 bp, *Methylosinus/Methylocystis*; 350 bp, *Methlyomicrobium* album; 58 bp, *pmoA/amoA*-like RA21 cluster; and 146 bp, *Methylosinus trichosporium* OB3B.

| *TRFs* | *47* | *58* | *80* | *113* | *146* | *245* | *350* |
|---|---|---|---|---|---|---|---|
| 47 | | | | | | | |
| 58 | -0.06 | | | | | | |
| 80 | -0.12 | -0.15 | | | | | |
| 113 | 0.02 | 0.02 | .-0.24 | | | | |
| 146 | *-0.34 | **0.39 | -0.21 | ***0.62 | | | |
| 245 | 0.18 | *-0.32 | -0.01 | ***-0.50 | 0.60 | | |
| 350 | 0.12 | .0.26 | 0.09 | -0.18 | -0.04 | 0.20 | |
| 531 | *-0.30 | -0.21 | -0.04 | 0.21 | 0.21 | **-0.36 | -0.16 |

Signif. codes: '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1

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# 4. Spatial heterogeneity is reflected in the functioning and community composition of methanotrophs in the littoral zone of a boreal lake

Environmental Microbiology (in preparation)

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## 4.1 Abstract

Methanotrophs play a globally important role by consuming methane in the oxic part of soils and sediments. In lake ecosystems a major proportion of methane emission is from the littoral zone. However, spatial heterogeneity in the functioning and diversity of methanotrophs in littoral wetlands is poorly understood. A particulate methane monooxygenase (*pmoA*) based microarray coupled with geostatics was used to analyze spatial patterns of methanotrophs in the littoral wetland of an eutrophic boreal lake (Lake Kevätön, Eastern Finland). Differences in the community structure and activity of methanotrophs depending on the variable hydrological conditions in the wetland were demonstrated. The wettest area, comprising the highest pH and $CH_4$ consumption, had the highest abundance and species richness of methanotrophs. Type Ib fresh-water-cluster methanotrophs favoured the wet area, as shown by the *pmoA* microarray, *pmoA* clone libraries and quantitative PCR. In addition to water level, the contribution of plant diversity on methanotroph diversity is discussed.

## 4.2 Introduction

Methane ($CH_4$) has a 25 times higher global warming potential than carbon dioxide with a time horizon of 100 years (Solomon et al. 2007). Methane concentration in the atmosphere has more than doubled from pre-industrial time due to increased anthropogenic $CH_4$-emissions (e.g. rice fields, landfills, eutrophication of lakes) and decreased $CH_4$-consumption resulting from land-use changes (Solomon et al. 2007). Whether an environment acts as a sink or a source of $CH_4$ is determined by the balance of production of $CH_4$ by strictly anaerobic methanogenic archaea and consumption of $CH_4$ by aerobic methane-oxidizing bacteria, methanotrophs. In aquatic environments also anaerobic $CH_4$ oxidation has shown to take place (Hindricks et al. 1999, Boetius et al. 2000) but its role in soil ecosystems is unknown. Natural wetlands contribute over 23 % of global $CH_4$ emissions (Conrad 2009). In natural wetlands, aerobic methanotrophs, are restricted to oxic-anoxic boundary layers, where methane and oxygen gradients overlap. There they can consume greater than 90 % of the $CH_4$ produced in deeper anoxic layers (Oremland & Culbertson 1992).

Aerobic methanotrophs are divided taxonomically into two phyla: *Proteobacteria* and *Verrucomicrobia*. The former has been the subject of extensive study for many decades, whilst methanotrophic *Verrucomicrobia* are a relatively recent discovery (Dunfield et al. 2007, Islam et al. 2008). Within *Proteobacteria* phylum methanotrophs are divided into two classes, *Gammaproteobacteria* (families *Methylococcaeae* and *Crenotrichaceae* often referred to as type I methanotrophs) and A*lphaproteobacteria* (families *Methylocystaceae* and *Beijerinckiaceae* often referred to as type II methanotrophs) on the basis of phylogeny, physiology, morphology and biochemistry (Hanson & Hanson 1996, Trotsenko & Murrell 2008). Key enzymes in microbial $CH_4$ oxidation are methane monooxygenases (MMOs). There are two kinds of MMOs, soluble, the cytoplasmic MMO (sMMO) and the particulate, membrane-bound MMO (pMMO). The pMMO is present in all known methanotrophs with the exception of the facultative methanotroph *Methylocella* spp. (Dedysh et al. 2005), whereas the sMMO is found in a limited number of strains. The *pmoA* gene encoding the 27 kDa subunit of the pMMO has been widely used as a functional marker in molecular ecological studies to investigate methanotrophs (McDonald, et al. 2008).

Plants provide organic substrates (above-ground litter, root litter and root exudates) for methanogens. Many emergent wetland plants have large interior open

spaces, termed aerenchyma, within their culms, stems or petioles, through which they release $CH_4$ from sediments while transporting oxygen from the atmosphere to support respiration in the roots (Amstrong 1976, Bubier 1995, Bergström et al. 2007). Oxygen transported by aerenchymatous vascular plants maintains microbial methane oxidation in their rhizospheres (King 1996). Wetland macrophytes vary in their ability to support rhizospheric $CH_4$ oxidation, which can be a result of the variation in their $O_2$ transport capacity (Calhoun and King 1997, van der Nat and Middelburg 1998). However, there is too little data to show the dependency of methanotrophic diversity on the composition of wetland macrophytes.

Littoral zones of lakes have a high primary production (vascular plants) which fuels methanogenesis in the water-saturated soil. Littoral zones can contribute as much as 70 % of the total $CH_4$ release from lakes (Juutinen et al. 2003a, Bastviken et al. 2008). Littoral wetlands typically have a high spatial variation in soil quality, vegetation and hydrology, all of which can affect production, oxidation, transport and release of $CH_4$ to the atmosphere. In boreal littoral wetlands water level is high in spring after snowmelt and decreases gradually when temperature is rising and plants start to grow. Water level fluctuates also during intermittent periods of high rain or dry spells. Changes in the water level affect both $CH_4$ production and $CH_4$ oxidation, and thus $CH_4$ fluxes, primarily through the availability of oxygen (e.g., Mosaavi and Crill 1997, Kettunen et al. 1999).

Since littoral zones contribute significantly to $CH_4$ emission from lakes, it is important to understand the environmental parameters affecting the species distribution of methanotrophs and their activity in various sub-zones of littoral areas. Previous studies in littoral areas of boreal lakes have depicted links between $CH_4$ fluxes, vegetation, sediment quality and hydrological conditions (Juutinen et al. 2003ab, Kankaala et al. 2003). Activity and distribution of methanotrophs in wetlands and in the roots and rhizosphere of emergent macrophytes have also been evaluated (King 1994, Boon et al. 1996). However, there are only a few studies, none of which are from boreal regions, evaluating activity and diversity of methanotrophs in lake sediments (Pester et al. 2004, Rahalkar et al. 2009). To the best of our knowledge, there are no previously published studies of the spatial heterogeneity of methanotrophs in littoral wetlands. In this study, we describe the spatial heterogeneity in methane oxidation and diversity of methanotrophs in a boreal littoral wetland possessing different hydrological and botanical sub-zones. We assessed the methanotroph community using a *pmoA* based

microbial diagnostic microarray (Bodrossy et al. 2003, 2006; Stralis-Pavese et al. 2004), *pmoA* clone libraries and quantitative PCR (Kolb et al. 2003).

## 4.3 Results

*Weather conditions and soil physical and chemical characteristics*

The average air temperature in July 2006 was 0.6 ºC higher and that in August 2006 1.1 ºC higher than the respective long-term (1971 – 2001) average. The study summer 2006 was exceptionally dry i.e. precipitation in July was 47 cm and that in August 37 cm below the respective long-term average values. The study area was divided, a-priori, into three sub-sites according to different altitude and water table level: wet, intermediate and dry (Fig. 1, Table 1) (water levels of sub-sites were different during July 2006 (two timepoints within a month), $df = 14$, $F = 8.46$, $P < 0.01$). These sub-sites had variable vegetation and soil chemical characteristics. The wet sub-site had the highest concentration of total C, total N, $NO_3^-$ and $NH_4^+$ in the soil (Table 1). The total content of carbon and nitrogen as well as the concentration of mineral nutrients tended to decrease with soil depth. However, the 0-2 cm layer occasionally deviated from this pattern, having sometimes lower values than the 2-10 cm layer.

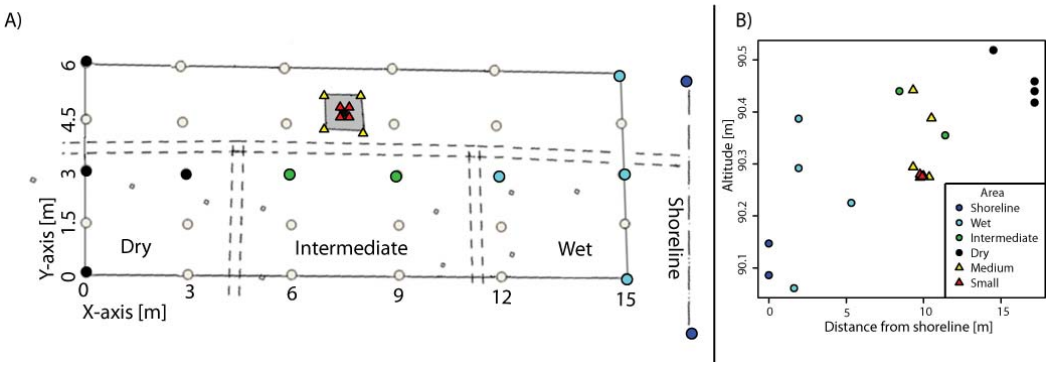


**Fig. 1.** a). Sampling scheme for spatial analysis of methanotrophs and positions of static chambers for $CH_4$ flux measurements. Large sampling scale: vertical side is 15 m, sampling interval is 3 m; horizontal side is 6 m, sampling interval is 1.5 m (sampling points, large open/colored circles (colored circles have been GPS-coordinated, Fig. 1B)). Medium sampling scale (grey box): both sides are 1 m, sampling interval is 0.25 m. Small sampling scale (black box): both sides are 0.25 m, sampling interval is 0.05 m. Chamber positions for the measurements of $CH_4$ fluxes are marked with *small open circles*. The large sampling plot was divided to dry (Y-axis 0-3 m), intermediate (6-9 m) and dry (12-15 m) sub-sites. Fig. 1B). Altitude of GPS-coordinated sites in spatial sampling area. GPS-coordinated points (GPS resolution was 3 cm) are colored in Fig. 1A) and their altitude (z-coordinate) is plotted into Fig. 1B) against distance from shoreline.

*Methane fluxes and oxidation potentials of $CH_4$ and kinetic parameters of $CH_4$ oxidation*

The methane oxidation as well as the fluxes of $CH_4$, followed the hydrological gradient being higher in wetter sub-sites ($F_{(methane\ fluxes)} = 37.80$, $P_{(methane\ fluxes)} < 0.01$; $F_{(methane\ oxidation)} = 381.94$, $P_{(methane\ oxidation)} < 0.001$) (Methane production potential also acted same way: wet > intermediate > dry (measured in September 2005, data not shown). The layers of the $CH_4$ oxidation potential experiments were different as well ($F = 160.74$, $P < 0.001$). The wet sub-site (closest to the shoreline) had the highest $CH_4$ emissions (15 mg $CH_4$ $m^{-2}$ $h^{-1}$) (Table 1) and $CH_4$ oxidation potential (Fig. 2), whilst the dry sub-site had low $CH_4$ emissions as well as low $CH_4$ oxidation potentials. In contrast to the wet sub-site, the dry sub-site acted occasionally as a net sink for $CH_4$. In addition that methane fluxes correlated with different subsites they were also connected with water table level ($F = 23.2$, $P < 0.05$)

Methane oxidation potentials were the highest in the uppermost organic soil layers (0-10 cm). The potentials were different in the soil layers ($P < 0.001$) and decreased with depth becoming negligible below 30 cm depth (data not shown). In the surface layer (0-2 cm) of the wet sub-site, the $V_{max}$ of the $CH_4$ oxidation was 403 nmol $CH_4$ $cm^{-3}$ $h^{-1}$ and the *K*m was 4300 ppmv (gas phase concentration). The $V_{max}$ and *K*m values for the surface layer of the dry sub-site were 199 nmol $CH_4$ $cm^{-3}$ $h^{-1}$ and 6600 ppmv, respectively.

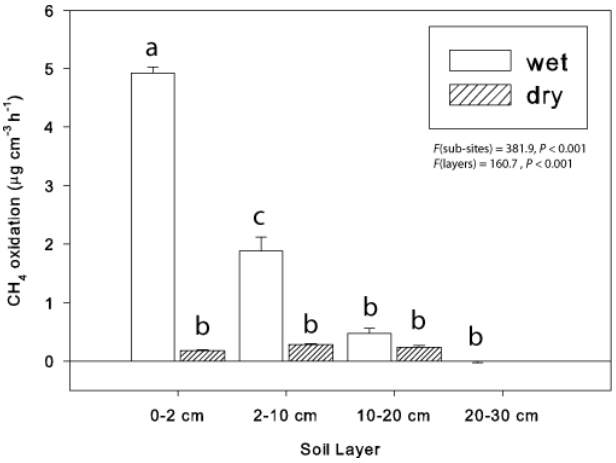


**Fig. 2.** Methane oxidation potentials measured in soil slurries. Means are from four replicate samples. SE of the means is shown by error bars. Variation was analyzed with two-way ANOVA (R 2.9.2) and paired comparisons between sub-sites were done with Tukey *post hoc* test. Significant differences are shown with letters ($P < 0.001$).

**Table 1.** Physical and chemical characteristics of soil samples taken in July 2006 from various distances to the shoreline (wet: 2 m, intermediate: 8 m and dry: 17 m from the shoreline).

| **subsite** | | Tot C[b] | Tot N[b] | pH ($H_2O$)[c] | Bulk density | Organic matter[d] | Water content[e] | $NO_3^-$ N[f] | $NH_4^+$ -N[g] |
|---|---|---|---|---|---|---|---|---|---|
| soil layer[a] | | % | % | | g $_{d.w.}$ $cm^{-3}$ | % | % | µg $cm^{-3}$ | µg $cm^{-3}$ |
| **Soil layers, July 2006** | | | | | | | | | |
| ***Wet area*** | | | | | | | | | |
| Water level[a] | -8 | | | | | | | | |
| $CH_4$ mg $m^{-2}$ $h^{-1a}$ | 15 | | | | | | | | |
| 0-2 cm | | 19 | 1.0 | 6.3 | 0.20 | 40 | 380 | 17 | 3.0 |
| 2-10 cm | | 22 | 1.2 | 6.5 | 0.20 | 41 | 405 | 0.87 | 6.1 |
| 10-20 cm | | 15 | 0.78 | 5.8 | 0.22 | 35 | 382 | 0.13 | 7.0 |
| 20-30 cm | | 5.9 | 0.34 | 5.5 | 0.71 | 7.7 | 78 | 0.01 | 16 |
| ***Intermediate area*** | | | | | | | | | |
| Water level | -14 | | | | | | | | |
| $CH_4$ mg $m^{-2}$ $h^{-1}$ | 4 | | | | | | | | |
| 0-2 cm | | 24 | 1.5 | | | | | | |
| 2-10 cm | | 10 | 0.72 | | | | | | |
| 10-20 cm | | 4.1 | 0.30 | | | | | | |
| 20-30 cm | | 3.8 | 0.27 | | | | | | |
| ***Dry area*** | | | | | | | | | |
| Water level | -25 | | | | | | | | |
| $CH_4$ mg $m^{-2}$ $h^{-1}$ | 0.3 | | | | | | | | |
| 0-2 cm | | 20 | 1.2 | 5.8 | 0.13 | 59 | 182 | 0.10 | 5.4 |
| 2-10 cm | | 6.9 | 0.53 | 5.5 | 0.29 | 21 | 94 | 0.09 | 1.4 |
| 10-20 cm | | 1.0 | 0.09 | 5.6 | 0.72 | 6.7 | 40 | 0.26 | 0.72 |
| 20-30 cm | | 0.55 | 0.04 | ND[h] | ND | ND | ND | ND | ND |
| **0-2 cm, August 2006** | | | | | | | | | |
| ***Wet area*** | | | | 5.3 | 0.21 | 49 | 368 | 1.5 | 9.2 |
| ***Dry*** | | | | 5.5 | 0.12 | 36 | 114 | 0.69 | 3.0 |

[a] The depth of the soil layers and water level (negative means below the soil surface) has been measured from the soil surface. The groundwater levels and $CH_4$ emissions are averages of measurements done on 4 Jul 2006 and on 24 Jul 2006 (3 replicate chambers/groundwater tubes per area). [b] Duplicate analyses were done with an elemental analyzer (samples were taken in October 2006 ) [c] Triplicate soil slurries consisted of 30 ml soil and 50 ml milliQ water [d] Four replicate analyses were made by igniting dry soil for 2 hours at 550°C [e] Duplicate analyses were made by drying soil at 65°C until the weight was constant, calculated as g $H_2O$ $g^{-1}$ dry soil *100 [f] Triplicate soil-water extracts (30 ml soil and 100 ml milliQ water) were analyzed with an ion-chromatograph, [g] Triplicate soil-KCl extracts (30 ml soil and 100 ml 1 M KCl) were analyzed with spectrophotometry [h] ND=not determined

*Spatial heterogeneity of methanotrophs*

Two aspects of the spatial variability of methanotrophs were considered. Firstly, the general spatial heterogeneity in the community structure of methanotrophs in the littoral wetland was analyzed. Secondly, the abundance of methanotrophs and community composition at the various sub-sites were evaluated.

The geostatistical analyses were performed at three different scales based on the size of the sampling plots: large (15 m x 6 m), medium (1 m x 1 m) and small (0.25 m x 0.25 m) (Fig. 1). Variograms at the large scale showed a broader spatial structure and higher range of spatial autocorrelation (Fig. 3A) than those at the medium and small scales (Figs. 3B, C). The range of autocorrelation was higher at the large scale (1.471 m) than at the medium and small scales (0.176 m and 0.033 m, respectively). The small sampling plot had the lowest total variance (Fig. 3C) and no distinguishable spatial structure. The directional semivariogram (Fig. 3D), estimated from the large scale data, indicates that the variation in the community structure of methanotrophs was higher (0.071) and community structure was more patchy (range 1.023) towards the direction of water level gradient (NW-SE) than along the shoreline (SW-NE) (0.052, range 1.273). In the medium and small sampling scales, the direction of the variogram had no effect on the total variation (data not shown).

Species distributions in the main groups of methanotrophs were visualized by using spatial interpolation, so called Kriging maps (Fig. 4). Kriging maps indicated that distribution of type Ib methanotrophs (Fig. 4C) showed the same pattern as the water table and they had the highest contribution to the diversity change in the large scale. According to the microarray data, type Ib methanotrophs were detected only in the wet sub-site on large sampling scale, whilst type Ia (*Methylobacter* sp., *Methylomonas* sp., *Methylomicrobium album* and *Methylosarcina*) and type II (*Methylocystis* sp., *Methylosinus* sp. and peatland related clones) methanotrophs were present in all sub-sites of the studied littoral wetland independently from the water table (Fig. 4A, B; Fig. S1). In smaller spatial sampling scales (medium and small plot), type Ib methanotrophs were detected also (Fig. S1). Compared to intermediate sub-site of large sampling scale these smaller sampling scales had lower altitude (Fig. 1B). The water table was measured from each sub-site beside collars of flux measurements. Water table was not measured particularly inside or closely around the medium and small sampling plot. But the altitude of the soil surface was evaluated with 3D-GPS (resolution 3 cm), and it shows that even though medium and small sampling plots are centered into the

sampling area, they are located by random into a small sump where the water level is potentially as high as it is in wet sub-site (Fig. 1B). Also the vegetation in medium and small plot is similar to wet sub-site in these plots showing the dominace of *Calla palustris* (Fig. S3, S4).

The microarray results indicated that the diversity change beyond type Ib methanotrophs was not due to *Methylococcus* or *Methylocaldum* species (not detected with microarray from wetland) but a change in members of an uncultivated group of type Ib methanotrophs (*Methylococcus*- and *Methylocaldum*-related freshwater sediment clones), so called fresh-water-cluster (targeted with microarray probes 501-286, fw1-286, LW21-374 and LW21-391) (Fig. 4C, D; Fig. S1). The Shannon-Weaver diversity index was higher in the wet sub-site (average H' 3.27) than in the intermediate (H' 3.05) or dry (H' 3.13) sub-sites ($F = 7.18$, $P < 0.005$).

The change in the methanotrophic community across the wetland was further analyzed with quantitative PCR (qPCR) to confirm the results obtained with the microarray. Quantitative PCR results indicated that type Ib and type Ia methanotrophs were more abundant in the wet site than in the dry site (Fig. S2), supporting the findings of the microarray (Fig. 4) and clone library analysis (Fig. 5). The abundance of type II methanotrophs was slightly higher in the dry site of the wetland. The moisture gradient affected ($F = 4.00$, $P < 0.05$) the total abundance of methanotrophs (type I + type II). The numbers were highest in the wet sub-site, average $3.02 * 10^5$ *pmoA* copies per gram of soil [dry weight], while intermediate and dry sites had 1.41 and $1.60 * 10^5$ *pmoA* copies per gram of soil respectively.

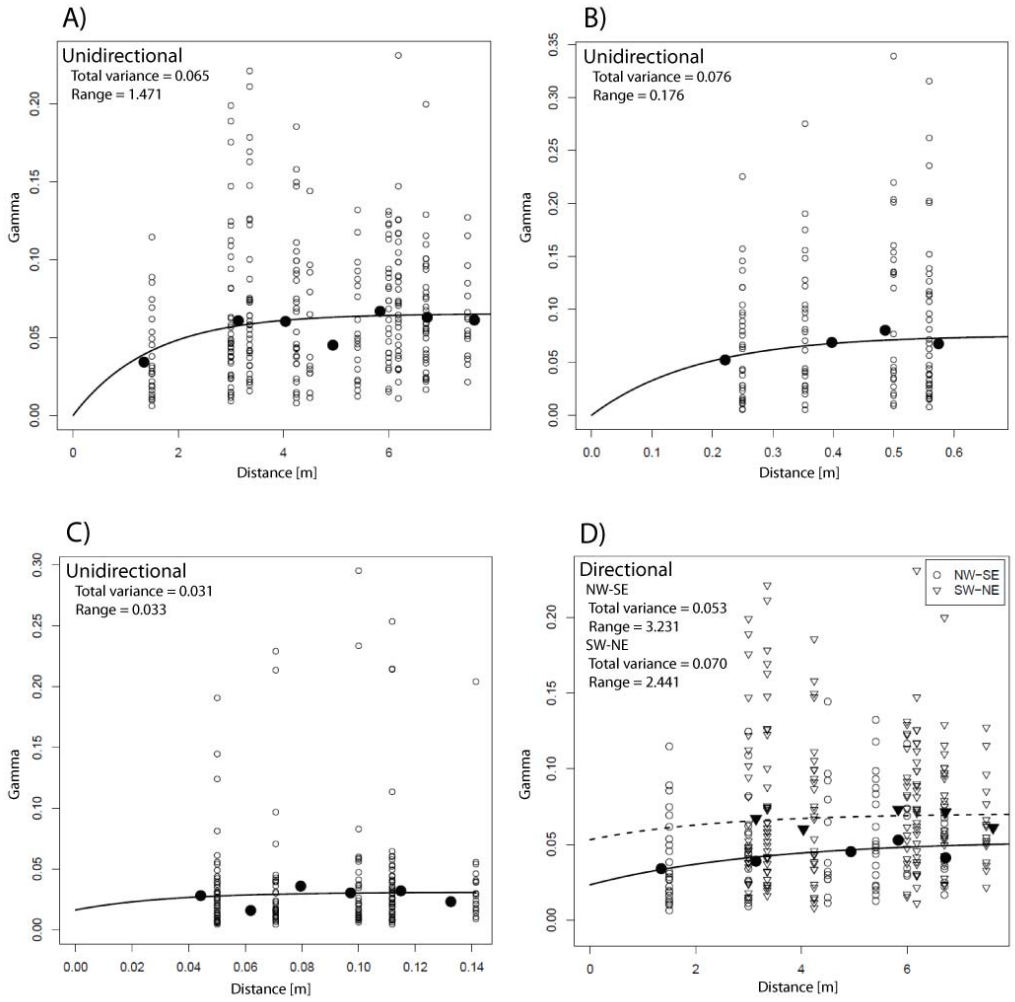


**Fig. 3.** Semi-variograms of large (**A**), medium (**B**) and small (**C**) sampling scale and directional semi-variogram (**D**) of large sampling plot. Filled symbols average semivariance of each distance group and open symbols square of distance of sample pairs.

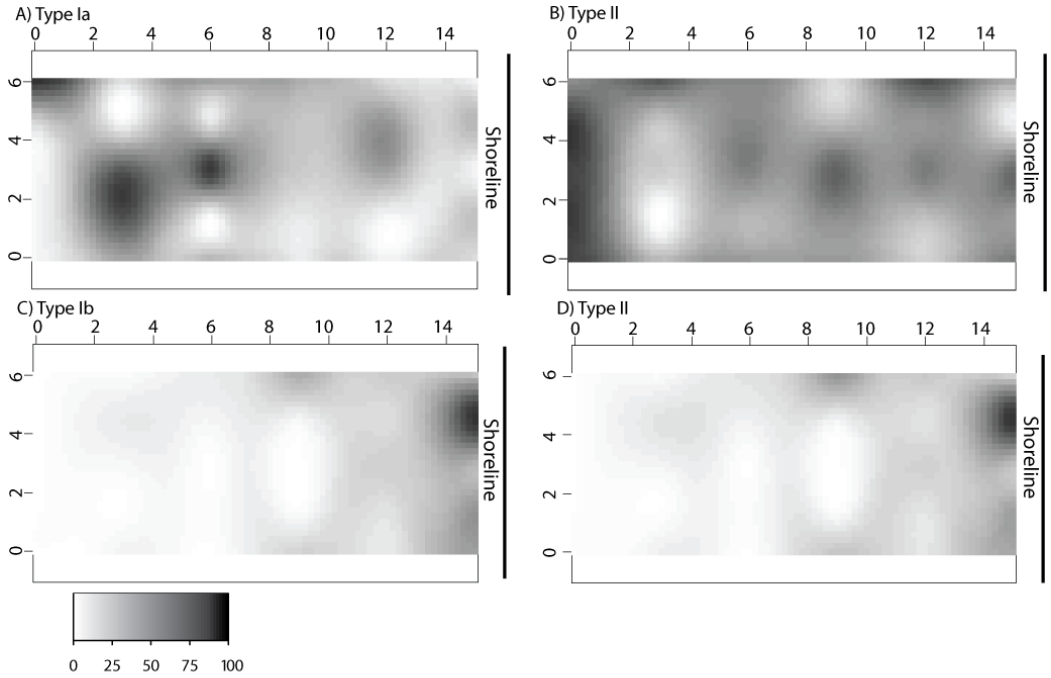


**Fig. 4.** Spatial interpolation maps for main groups of methanotrophs. Maps represent relative abundance of methanotrophs based on detection by *pmoA* microarray of Bodrossy and colleagues (2003). Analysis was done by reducing the highly multivariate microarray data for the studied types of methanotrophs into only two latent variables by using principal component analysis. Then spatial interpolation was applied to these latent variables in order to study both the spatial dependence and the spatial structure of the studied methanotroph types at the large sampling plot. A black color indicates maximum value (100 %) of principal component for each methanotroph group, while white color indicates minimum (0 %). Fresh-water-cluster = detected by probes targeting environmental clones from profundal and littoral sediment of Lake Constance and Lake Washington (Costello and Lidstrom 1999, Pester et al. 2004).

*Effect of plant diversity on the methanotrophic community structure*

The analysis of dissimilarities showed that, on the medium and small scale, diversity of methanotrophs was not directly associated with plant community structure. The heatmap of universal and fresh-water-cluster probes of small and medium scales shows variability expecially in activated freshwater phylotypes (detected with LW21-391) and in type II phylotypes (detected with II509) (Fig. S4). Even though direct correspondence between vegetation and methanotrophs was not possible to estimate, *Calla palustris*, typical vegetation of wet areas was showing higher (statistical not significant) appearance of type Ib methanotrophs (detected by universal microarray probe Ib453), whilst type II and type Ia (detected by II509, II530, Ia193 and Ia575) methanotroph species were growing better together with *Potentialla palustris and Calamagrostis canescens* (Fig. S4.).

*pmoA clone libraries*

The clone libraries were created with A682 and semi-nested (with mb661r) PCR strategies from surface soil (0-2 cm layer) of the wet and dry sub-sites of the wetland to cover the maximum hydrological variation of the wetland (Fig. 5A). The clone libraries showed high taxonomic richness.

The clone libraries from dry sub-site were dominated by type II phylotypes, (*Methylosinus, Methylocystis* and clones related) (Fig. 5B). Clones related to the peatland environmental clones were found both in the wet and dry sub-sites. Methanotrophs of the type Ia (*Methylobacter* sp. and *Methylomicrobium album* and *Methylosarcina fibrata* related clones (previously detected in rice paddies and fresh water environments)) also appeared in both sub-sites, but they were less abundant than the methanotrophs of the types II or Ib. Type Ia clones related to *Methylomonas* sp. and environmental clones from Soda Lake were also detected. Type Ib phylotypes occurred only in the clone library of the wet sub-site where they dominated over the type Ia phytotypes (Fig. 5B). Type Ib clones in libraries were mostly related to the uncultured fresh-water-clones (from Lake Constance and Lake Washington), but the closest relatives of *Methylococcus* or *Methylocaldum* were detected in very low numbers. Also in the wet sub-site *Crenothrix polyspora* –related sequences were detected.

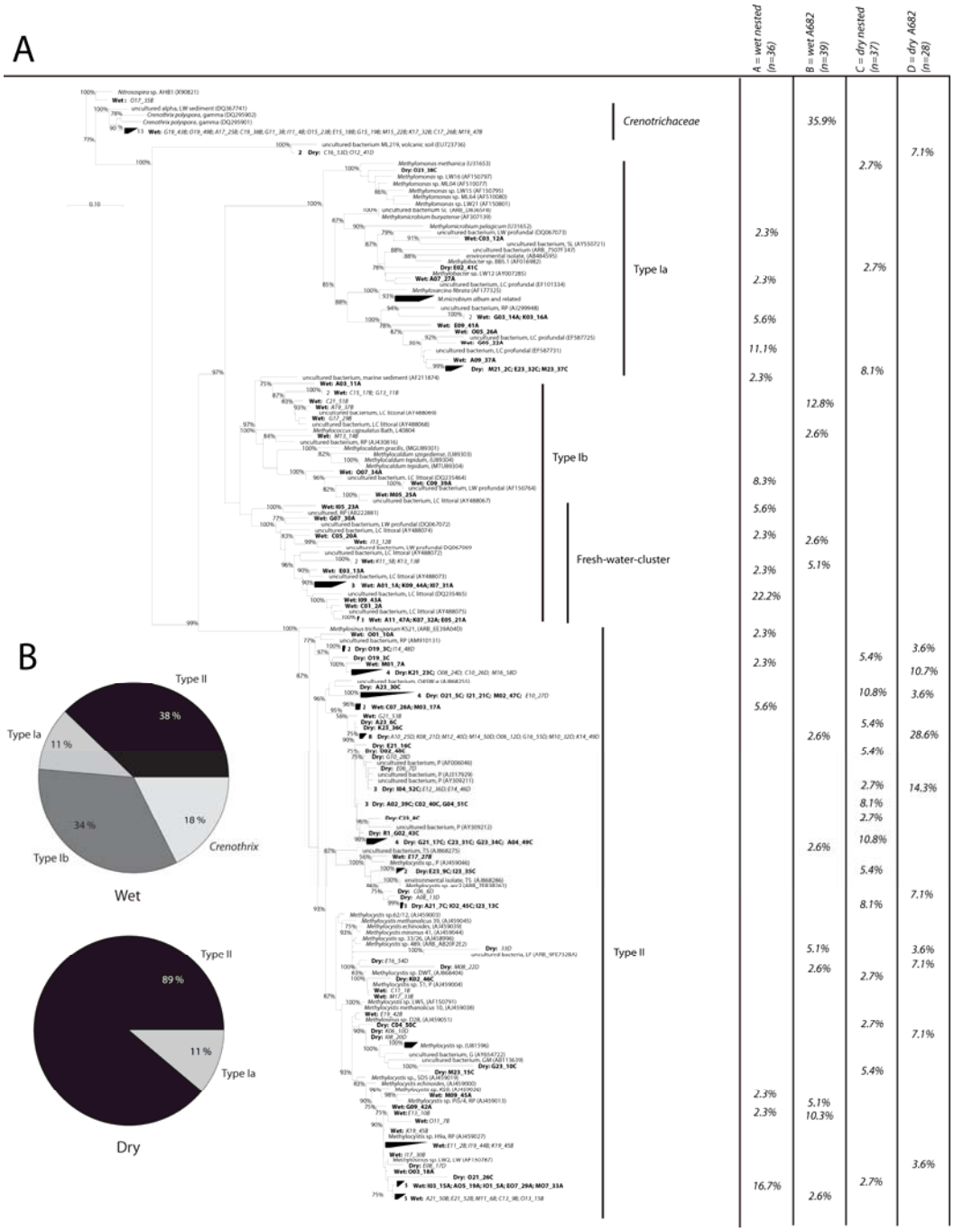


**Fig. 5.** A) Neighbour joining phylogenetic tree (1000 bootstraps) using a Felsenstein corrected distance matrix of *pmoA* sequences. Bootstrap values greater than 75 % are shown at the nodes. Frequencies of each clone groups of libraries from the wet sub-site (A = nested, B = A682r) and the dry sub-site (C = nested, D = A682r) is shown in sidebar. Abbreviations for origin of environmental clones: RP = rice paddy soil, LW = Lake Washington, LC = Lake Constance, SL = Soda Lake, LF = landfill, TS = tropical soil, P = peat, G = grassland, GM = gold mine. B) Taxonomical groups retrieved from clone libraries of the wet and dry sub-site.

## 4.4 Discussion

*Spatial heterogeneity of methane fluxes and functioning of methanotrophs*

The wet sub-site (closest to the shoreline) had the highest $CH_4$ emissions (Table 1) and potential for $CH_4$ production (results not shown) and oxidation. The $CH_4$ emissions in the wet sub-site were similar to those reported for boreal (Huttunen et al. 2003) and arctic (Joabsson et al 1999) wetlands in general. Methane emissions measured in this study were also on same level as reported previously by Juutinen and colleagues (2003b) from the same study site. Methane emissions were stimulated by high water table, which is supported by previous findings from wetlands (e.g., Bubier 1993, Mosaavi and Crill 1997, Christensen et al. 2003).

Oxygen could penetrate deeper into the soil in the dry sub-site than in the wet sub-site, due to the lower water table. However, $CH_4$ oxidation activity did not occur more deeply in the dry sub-site than in the wet sub-site, being negligible in soil layers below 30 cm, in both areas. Methane oxidation activity in the corresponding soil layers was always lower in the dry sub-site than in the wet sub-site. This could be due to the lower production of $CH_4$ in the dry soil, resulting in lower methane availability for methanotrophs. The water table gradient over sub-sites was clearly the dominant factor on the $CH_4$ production as well as $CH_4$ oxidation potentials. High nitrate concentration in surface layer of the wet sub-site could have had some negative effect (electron acceptor) on methane production.

The average affinity of methanotrophs for $CH_4$ was in the same range for the dry and wet sub-sites (*K*m; dry 6600 ppmv and wet 4300 ppmv), but the maximum $CH_4$ oxidation capacity was significantly higher in the wet sub-site ($V_{max}$ = 403 nmol $CH_4$ $cm^{-3}$ $h^{-1}$) than in the dry sub-site (199 nmol $CH_4$ $cm^{-3}$ $h^{-1}$). It is notable that the *K*m and $V_{max}$ values shown here are averages for the whole methanotrophic community, not for certain groups or species. Even though there was a difference in the maximum oxidation capacity between the dry and wet sub-sites, it cannot be determined whether this change is the result of the functioning of the whole methanotrophic community or a small sub-group. The methanotrophic community in the littoral wetland studied here has low affinity for $CH_4$. Methanotrophs in upland forest soils in temperate and boreal regions show *K*m values ranging from 5 to 92 ppmv (Bender and Conrad 1992, Whalen and Reeburgh 1996, Saari et al. 2004), and in low-affinity ecosystems (profundal sediments and peatlands) *K*m has reported to be 7900-43000 ppmv (Lidstrom and Somers 1984,

Watson et al. 1997). The maximum $CH_4$ oxidation capacity in the wet sub-site ($V_{max}$ 403 nmol $CH_4$ $cm^{-3}$ $h^{-1}$) was similar to that reported for the profundal sediment of Lake Washington ($V_{max}$ 346 mol $CH_4$ $cm^{-3}$ $h^{-1}$) (Auman et al. 2000), but the $CH_4$ oxidation potential here was much higher (the highest value in the surface soil was 110 nmol $CH_4$ $cm^{-3}$ $h^{-1}$) than that reported for the littoral sediment of Lake Constance, 0.18 nmol $CH_4$ $cm^{-3}$ $h^{-1}$ or for the water column of Mono Lake, 5.13 pmol $CH_4$ $cm^{-3}$ $h^{-1}$, (Rahalkar et al. 2009, Carini et al. 2005).

*Spatial heterogeneity of methanotrophs*

Geostatistical analysis indicated that spatial factors affect the methanotrophic community in the littoral wetland. The large-scale analysis indicated that the spatial pattern of the methanotrophic community was organized with a range of 1.471 m. Variograms suggest that the methanotrophic community has a large-scale heterogeneity (according to the nomenclature by Ettema and Wardle 2002) over the littoral wetland. The methanotrophic community in the littoral wetland is thus more patchy than that in rice paddies (Krause et al. 2009) or in landfill cover soil (Kumaresan et al. 2009). The patchiness of the methanotrophic community in the littoral wetland also exceeded that of the denitrifier communities under different cattle impacts in grassland (6-16 m range of autocorrelation, Phillippot et al. 2009) and the microbial communities in forest soil (20 m range of autocorrelation, Saetre and Bååth 2000). In addition to environmental factors (i.e. water table, soil texture), also taxonomic resolution is affecting on observations of spatial heterogeneity. In fact comparison to studies done with gene markers is more accurate than studies done with PLFA patterns. Even though experimental set ups are not always similar in different studies of spatial heterogeneity and ranges of autocorrelation cannot by compared directly, this study suggests that fluctuations in the water table result in patchiness of the methanotrophic community in the littoral wetlands.

The water table gradient is the result of the geomorphology of the littoral wetland, and this gradient clearly affects methanotrophs there. The water table controls the availability of oxygen and $CH_4$, and thus the presence and activity of methanotrophs as well as their spatial variability. The spatial variation of methanotrophs was higher in the direction of the water table gradient than along the shoreline, as demonstrated by the directional variogram. The water level controlled the abundance of different types of methanotrophs. Type Ia (*Methylobacter*, *Methylomicrobium*, *Methylomonas* and

*Methylosarcina* species) and type II (*Methylocystis*, *Methylosinus* species) methanotrophs appeared in all sub-sites (wet, intermediate and dry) of the wetland studied. The microarray data indicated that the moisture gradient did not affect cell numbers of type Ia methanotrophs and they seem to be generalists in the wetland environment. Type II methanotrophs have been shown previously to react more slowly to changes in $CH_4$ availability than type I methanotrophs (Henckel et al. 2000). This data suggest, that not only type II display a typically slow response but also that type Ia in wetland environments seems to use this same lifestyle. The abundance of type II methanotrophs, shown by clone libraries and qPCR, was higher in the dry sub-site. It appears that type II methanotrophs survive better in dry conditions. On the other hand, *Methylocystis* spp. can form drought-resistant cysts (Whittenbury et al. 1970) which can survive for long periods in unfavourable conditions (Rothfuss et al. 1997). In dormant cells such as cysts, DNA can be stable in soil (Lindahl 1993). Therefore, community analysis performed at the DNA level may not give accurate information about the active methanotroph population. Study of a marine estuary sediment demonstrated type II methanotroph phylotypes, despite being present in the DNA, were inactive, whereas type I methanotrophs were active in the sediment (Moussard et al. 2009). Both the microarray and the clone library analysis indicate that water-saturated conditions support the growth of the type Ib fresh-water-cluster methanotrophs. To the best of our knowledge, this is the first study in which soil moisture has been shown to determine the presence of this group of methanotrophs.

The methanotrophic community in the wet sub-site also had a higher $V_{max}$ value than the dry sub-site, indicating that either there are more methanotrophs in the wet sub-site or they have higher specific activity (oxidation rate/cell). In the wetland, both the species richness and the abundance of type I and type II methanotrophs were higher in the wet sub-site than in the dry sub-site. The higher average water table in the wet site favours $CH_4$ production, and this subsequently increased substrate availability supports the activity of methanotrophs. In the wet sub-site, the higher water table causes the higher methane oxidation rate, higher species richness, higher abundance of methanotrophs and appearance of type Ib related fresh-water clones. Soil pH was also highest in the wet site but it is unclear if this has an effect, in addition to the water table, on methanotrophs. Previous studies have indicated that higher species richness is coupled with high pH (Fierer and Jackson 2006) and $CH_4$ oxidation potential (Lin et al. 2005). In addition, wetlands are suitable habitats for protistan grazing (Murase et al.

2006). Protozoa are known to prey preferentially on type I methanotrophs (Murase and Frenzel 2008). Grazing may control the community of methanotrophs and their activities especially in the wet parts of littoral wetlands which are inhabited by type I methanotrophs.

Type Ib methanotrophs were appearing in large scale plot only in wet side of the area, but they also appear in medium and small scale plots. In these smaller plots the altitude was at same level as in wet sub-site. If the geomorphology of wetland would be more homogenous and the altitude would be linearly decreacing when reaching the shoreline (and altitude of small and medium plot would be at same level with surrounding intermediate sub-site), the appearance of type Ib methanotrophs in these smaller plots would hinder making such a conclusion that water level is factor controlling the appearance of type Ib methanotrophs. Now, the appearance of type Ib in smaller plots confirms their dependency on high water table.

The geostatistical analysis indicated that there was also spatial variation in the methanotrophic community at the medium and small scales. On these smaller scales, the water level was more constant than large scale and in such a small area, constant factors as different plants are, may affect more on microbial communities than water table fluctuation itself. Therefore, effects of plants on the methanotrophs could be evaluated in such area. Plants are known to affect microbial diversity in general (Kowalchuk et al. 2002, Loranger-Merciris at al. 2006). Even though there was variation in methanotroph community in small and medium scales the correlation between vegetation and methanotroph community cannot be found. The results demonstrate a slight correlation but variation cannot be explained with above ground vegetation. Root-architecture of plants differs and they could also overlap each others and cause various effects on methanotroph community. Heatmap results showed that there was higher amount of freshwater-cluster clones and some species of type II methanotrophs in certain areas of medium and small scale plot. However, the present data does not allow to draw a conclusion about dependency between vegetation and methanotroph diversity in littoral wetland. The effect of plant species on methanotroph diversity could be further studied with a laboratory experiment with stable water table conditions.

In conclusion, we demonstrate that the water level causes large scale variation in the activity and diversity of methanotrophs in the littoral wetlands. Plant diversity may further affect the community composition of methanotrophs. It remains to be

demonstrated what the causal mechanisms are by which water table, soil chemical characteristics and vegetation affect activity and diversity of methanotrophs.

## 4.5 Experimental procedure

*Study site*

Soil samples were collected from the temporally flooded littoral wetland of Lake Kevätön in Eastern-Central Finland (63°6´N, 27°37´E). Kevätön is a hyper-eutrophic, relatively small (4 $km^2$) and shallow (mean depth 2.3 m) lake and it is ice-covered from mid November to early May. In the study site the long-term (1971-2001) average air temperature and rainfall during summer (June-September) are 13.9 °C and 70 mm, respectively.

The littoral area has a gradient with changing moisture (Table 1) and vegetation. In the wettest sub-site dominant plant species were *Calla palustris*, *Carex aquatilis* and *Potentialla palustris,* while the dry sub-site was dominated by *Calamagrostis canescens.* During spring the study site is flooded, but during the summer, when measurements were taken, the water level was below the soil surface. The width of the studied transect was six meters and the length was fifteen meters. The depth of the organic layer of the soil was 20 cm in the wet sub-site and 10 cm in the dry sub-site (Table 1). Weather conditions were recorded at the meteorological station 15 km from the study area. The coordinates and altitude of the study area (corners of spatial sampling plots and sampling intervals with different distances from shoreline) was measured with 3D-GPS (resolution 3 cm) (Fig. 1B).

*Soil sampling*

Triplicate soil cores (depth ≈ 40 cm) for determining $CH_4$ oxidation potential, clone library for methanotrophs as well as soil physical and chemical characteristics were taken in July 2006 by using a box corer (8 x 8 cm) from within a 1 x 1 m site adjacent to the sample sites with co-coordinates (Fig. 1, wet: Y=0, X=3 and dry: Y=15, X=3). In the field, sediment profiles were divided into layers of 0-2 cm, 2-10 cm, 10-20 cm and 20-30 cm (depth from the soil surface). Samples for molecular biological analyses were frozen with dry ice in the field before storing them at -80 °C.

Samples for determining the spatial heterogeneity of methanotrophic community were taken (a corer, i.d. 3 cm) in August 2006 from the surface organic layer (0-2 cm) known to have the highest $CH_4$ oxidation activity. Then in total, 80 samples (Fig. 1A) were taken from three nested sampling plots: large (15 x 6 m, 30 samples), medium (1 x 1 m, 25 samples from regularly spaced grid with 25 cm xy-intervals) and small (0.25 x

0.25 m, 25 samples from regularly spaced grid with ≈ 6 cm xy-intervals). At the same sampling time also surface soil (0-2 cm) was collected from wet and dry sub-site for analysis of kinetic parameters of $CH_4$ oxidation.

Since the variation of vegetation was highly variable over the study area, the plant diversity of four dominant plant species (*Calla palustris*, *Carex aquatilis*, *Calamagrostis canescens*, *Potentialla palustris)* was evaluated. In the large scale sampling plot (15 x 6 m), the aboveground plant community was visually evaluated around (in area with diameter of 50 cm) each spatial sampling site where the soil core (diameter 3 cm) were collected. In medium and small sampling plots, where the sampling distances were shorter only the present plant in each sampling sites were documented.

*Physical and chemical analyses of soil*

During methane flux measurements, sediment and air temperatures and water levels were measured beside each collar. Temperature of the soil was measured at depths of 0, 2, 5, 10, 20, and 30 cm using a Fluke 51 K/J thermometer. Water level was measured from perforated PVC wells beside each collar.

Soil water content was determined by drying duplicate sediment samples at 65 ˚C. Organic matter content was determined by igniting dry homogenized soil samples at 550 ˚C. Soil pH was determined from water-soil suspension (25 ml soil and 100 ml milliQ-water) by using a WTW pH320 meter.

Nitrate and ammonium were extracted from triplicate samples from soil layers of 0-2, 2-10, 10-20, and 20-30 cm. For nitrate, a mixture of 25 ml soil and 100 ml milliQ-water were shaken for 1 hour at 175 rpm and filtered through 589/3 ashless, blueribbon filter paper circles (185 mm) (Schleicher & Schuell). Ammonium was extracted similarly except that milliQ-$H_20$ was replaced with 1 M KCl. The extracts were stored at -20 ˚C prior to analysis. Nitrate were analyzed using a Dionex DX-120 ion chromatograph equipped with an AS 9-HC 4 mm anion column, an ASRS Ultra 4 mm suppressor (Dionex Co, USA) and autosampler (AS40). Ammonium was determined spectrophotometrically (Fawcett and Scott 1960).

*Methane fluxes*

Methane fluxes were measured *in situ* with a static chamber technique twice in July 2006. 10 collars, i.e. 60 x 60 cm aluminium frames had been embedded into the surface

soil (three for the wet sub-site, four for the intermediate sub-site and three for the dry sub-site). Flux measurements were performed from the boardwalks besides the collars to minimize disturbances. Before each flux measurement the water grooves of the collars were filled with water to ensure gas-tight closure of the chamber. Headspace height varied from 40 to 120 cm depending on the height of vegetation. The chambers had a fan to mix the air in the chamber and a thin ventilating tube to prevent low pressure during gas sampling. Five gas samples (40 ml) were taken in a 25 min measurement period with 60 ml polypropylene syringes (Terumo) equipped with tree-way stopcocks (Connecta). Methane samples were analyzed within 24 h of sampling with a gas chromatograph. Methane fluxes were calculated from the linear increase (or decrease) in the headspace gas concentration with time.

*Methane oxidation activity and kinetic parameters of $CH_4$ oxidation*

Methane oxidation was studied by incubating sediment slurries, consisting of 25 ml of sediment and 50 ml of distilled water, in 550 ml flasks on a rotary shaker (175 rpm) at 15 ºC in the dark. At the beginning of the experiment $CH_4$ the headspace of the flasks had 0.5 % $CH_4$ and an air overpressure of 20 kPa. Methane samples (15 ml) were taken five times during the incubation of 48 hours. First-order reaction constants for $CH_4$ oxidation were determined from the ln-transformed decrease in the $CH_4$ concentration over time and normalised to $cm^{-3}$ soil. Methane oxidation rates (ng $cm^{-3}$ $h^{-1}$) were calculated by multiplying the reaction constants with the corresponding initial headspace concentration of $CH_4$.

Kinetic parameters of $CH_4$ oxidation were determined as $CH_4$ oxidation in various soil layers except that soil slurries consisting of 5 ml surface soil (0-2 cm) and 30 ml of distilled water were incubated with variable initial headspace concentrations: 0.03, 0.06, 0.1, 0.5, 1, and 2 %) Flasks with an initial headspace concentration of 0.5 % $CH_4$ or more had a 2-3 days lag phase before $CH_4$ concentration started to decrease. Methane oxidation was calculated from the active part of the $CH_4$ oxidation curve. Lineweaver-Burk plots were used for determining $V_{max}$ and $K_m$.

*Methane analyses*

Methane samples were analyzed within 24 hours of sampling. Gas samples from $CH_4$ oxidation experiments and chamber measurements were analyzed with HP 5890 Series II by injecting gas via 0.5 ml loop. Both gas chromatographs were equipped with a

flame ionization (FI) detector for $CH_4$ and a Hayesep Q 80/100 mesh column (length 1.8 m) The detector and oven temperatures were 150 ºC and 35 ºC. Two standards were used for $CH_4$, 1.98 ppm for lower concentrations (fluxes and oxidation incubations) . The sample gas was passed through a glass tube filled with $P_2O_5$ to remove water vapour before entering the GC. Gas analyses have been described in more detail by Nykänen et al. (1995).

*Soil DNA extraction*

DNA was extracted with bead beating techinique by using FastDNA SPIN kit for soil (Q-BIOgene) and phenol/chloroform extraction based on minorly modified protocol published by Stralis-Pavese et al. (2004). One hundred mg of freeze-dried mortar-homogenised soil (stored at -70 ºC) was used for DNA extraction. The lysis buffer was modified by adding 2 % w/v PVP K30 (Fluka). After purification, DNA was eluted with 50 µl TE (pH 8.0) and stored at -20°C.

*pmoA clone library construction and phylogenetic analysis*

Clone library analysis was performed on DNA extracted from the surface layer (0-2 cm, $CH_4$ oxidation potential is the highest) of the dry and wet of the study site. Touchdown PCR was performed with A189f + A682r (Holmes et al. 1995) (35 cycles, first 11 cycles with annealing 62°C -1°C per cycle until 52°C is reached, followed by 52°C annealing for 25 cycles) PCR strategy and semi-nested gradient two-step PCR strategy, first round with A189f + A682r primers (35 cycles, first 11 cycles with annealing temperature 62°C -1°C per cycle until 52°C is reached, followed by 52°C annealing for 24 cycles) followed by a189f + mb661r primers (Costello and Lidstrom 1999) (25 cycles, first 11 cycles with annealing temperature 62°C -1°C per cycle until 52°C is reached, followed by 52°C annealing for 14 cycles), as reported previously (Moussard et al. 2009) with exception that 50 ng of DNA were used for amplification of *pmoA* genes. PCR products were purified with HighPure PCR purification kit (Roche Diagnostics GmbH) and ligated into the pDRIVE vector (Qiagen). DNA sequencing was performed using Applied Biosystems 3730XL automated sequencing system by DNA sequencing service (Macrogen Ltd, Seoul, Korea). Sequences were imported into a database of >4000 pmoA sequences (both publically available and private) using the PT-server fast alignment function in ARB, then corrected manually. Subsequently a bootstrapped (1000 replicates) neighbour joining tree was constructed using Felstein

correction in the ARB software package (Ludwig et al. 2004). Sequences from this study have been submitted to the EMBL database under accession numbers FN597111-FN597251.

*Methanotroph community analysis with pmoA-microarray*

The microarray construction and the set of oligonucleotide probes used in this study are described by Bodrossy and colleagues (2003) and Abell and colleagues (2009). Targets for *pmoA* microarray were generated with same PCR protocol as decribed previously (Moussard et al. 2009). Target were amplified with the same two-step semi-nested PCR protocol as used for clone library construction with an exception that the A682r and mb661r primers had a 5' T7 recognition site. Target labelling, hybridization and scanning were performed as described previously (Stralis-Pavese et al. 2004).

*Quantitative PCR*

Quantitive PCR analysis of *pmoA* gene was done according to previously published method (Kolb et al. 2003) for three different group of methanotrophic bacteria: *Methylobacter* and *Methylosarcina*, *Methylococcus* and *Methylocaldum, Methylosinus* and *Methylocystis*. Specificity of primers were checked against *pmoA* clone library *in silico*. PCR reactions were performed in 20 µl volumes in triplicate. The reaction contained 500-fold diluted Sybr Green (Invitrogen), 1x PremixF mastermix (Epicentre), 0.25 µM of each primer, 1 U *Taq* recombinant polymerase (Invitrogen) and 10-20 fold diluted DNA solution. Quantification of each group was calculated with a stardard curve using 10-fold diluted standard series of perfect matching clone from *pmoA* clone library as a control. Presence of PCR inhibiting substances in DNA was tested by dilution serie of samples. It was shown that 10-20 fold diluted DNA from littoral wetland did not inhibit qPCR reactions, with a linear relationship between target dilution and Ct value present across the range of template concentrations. Average values for abundance of methanotrophs (type I + type II, excluding *Crenotrichaceae* and *Beijerinckiacaea*) in each sub-sites of large sampling plot were calculated with spatial study samples (layer 0-2 cm) from dry (average of all samples in sampling grid (Fig. 1A) with Y-axis values 0-3 m), intermediate (Y-axis values 6-9 m) and wet (Y-axis values 12-15 m) sub-sites.

*Statistical analysis*

Measured variables of production, oxidation as well as the fluxes of $CH_4$ from sub-sites where evaluated with two-way analysis of variance (ANOVA) and paired comparisons were done with Tukey *post hoc* test. Prior to the analysis, normality of the variables was analyzed with Shapiro-Wilk test. All populations differing significantly from normal distribution were log transformed. Tests were done with a statistical program R 2.9.2 (R Development Core Team 2009).

For analysis of spatial heterogeneity of methanotrophs, geostatistics (e.g. Cressie 1993) was used. Geostatistics originates from geological science and the method has been used for predicting e.g. mineral resources. Nowadays it is used also in ecology for analysing spatial patterns of plant, animal and microbial communities. The analysis is based on the assumption that locations close to each other are more similar than those further apart. The dependency of measurements with different sampling distances is calculated as semi-variance $\gamma(h)$, which quantifies the amount of variation observed at specific distance *h*. If the variation depends on distance, i.e. the variation within the study area is not completely random, spatial autocorrelation can be observed and the semi-variance can be plotted against spatial distance in order to get a variogram. The slope of the variogram indicates the type of the observed heterogeneity (Ettema and Wardle, 2002).

The binned semivariance values $\gamma(h)$ were calculated from the Bray-Curtis distances of original semi-quantitative microarray data. The binned variograms were calculated using the Hawkins and Cressie's modulus estimator (Cressie 1993) due to the non-normality of the data sets and exponential models were fitted to the $\gamma(h)$ values since they seemed to fit and since this choice made the interpretation of the parameter values easy.

The representation of spatial structure in methanotroph assemblages was made by spatial interpolation maps (so-called Kriging) across the large scale plot of each different type of methanotrophs using the R add-on package geoR (Ribeiro & Diggle 2001). In these analyses second degree polynomial trend was taken into account and directional (NW-SE and SW-NE) semivariograms were created. Also here the modulus estimator was used, but instead of the simple exponential model, more flexible cubic variogram models with a fixed nugget value of zero were fitted to the $\gamma(h)$ values. The Kriging maps were created using ordinary kriging supplemented by estimated trend. To

calculate the $\gamma(h)$ values and to plot the variograms and kriging maps the R 2.9.2 was used.

Richness of methanotrophic community in different subareas of large sampling plot was analyzed with Shannon-Weaver diversity indices. Richness was calculated with the *vegan* (Oksanen et al. 2008) add-on package in R 2.9.2. The effect of distance to shoreline was tested by evaluating dissimilarities of sample groups having different distance from shoreline. This was done with *adonis* function in R add-on package *vegan*. On the medium and the small sampling scales, where the water table is rather constant, the effect of plant diversity on methanotrophic community was analyzed. To evaluate the effect of plant diversity on the methanotrophic community, samples in these plots were devided into to a groups according the vegetation. The effect of vegetation was tested by analysing dissimilarities of vegetation groups with *adonis* function. Vegetation effect on each *pmoA*-microarray probe was also compared with non-parametric Kruskall-Wallis test. Tests and hierarchically clustered heatmap visualization of universal and fresh-water-cluster probes were done with the *gplots* add-on package in R 2.9.2.

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## 4.7 Supplementary information

**Supplementary Methods:**

*Statistical analysis*

Semivariance value in geostatistical analysis is simple to calculate for univariate datasets but in the case of microbiology the multivariate data needs to be converted into multivariate distances. Traditionally this has been done by using dissimilarity matrix (Franklin et al. 2002), components obtained with principal component analysis (Saetre & Bååth 2000) or Jaccard´s coefficient index calculated binary data (Franklin & Mills 2003). For geostatistical analysis two different approaches to generate variograms were tested: pseudovariograms with Jaccard´s coefficient index (e.g. Franklin & Mills 2003, Krause et al. 2009) and semivariance $\gamma(h)$ –values calculated from original microarray data. This was done because we wanted to test whether the strategy of generating variograms from the original multivariate dataset would affect the final result. This test confirmed that both strategies could be used for generating variograms from microarray data and they produced similar results (data not shown). In this study, we chose to use semivariance $\gamma(h)$ to describe the spatial heterogeneity of methanotrophs.

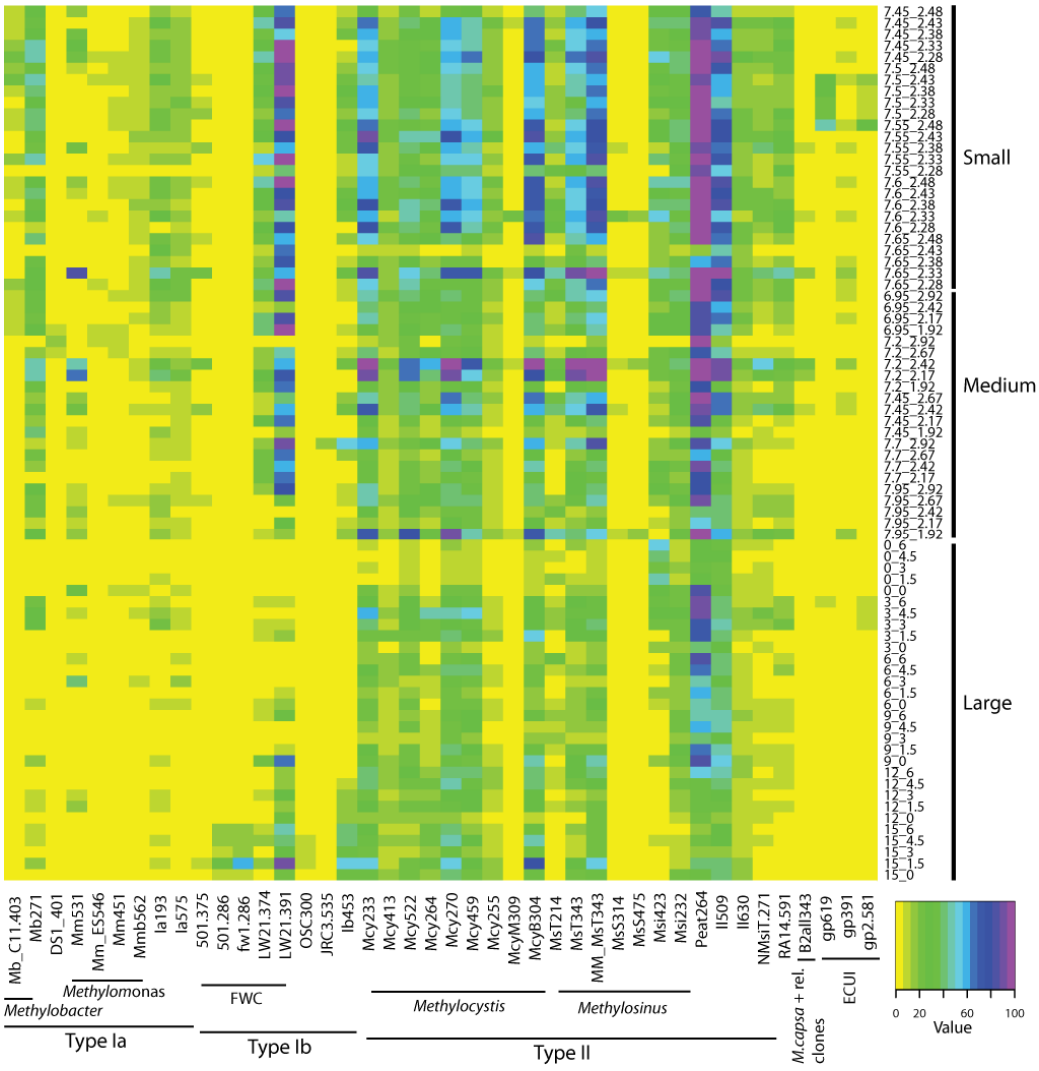


**Fig. S1.** Microarray results of spatial study showing the community composition of methanotrophs. Name of each sample shows coordinates of sample (x_y). ECUI = environmental clones with uncertain identity, FWC = Fresh-water-cluster. A value of 100 (purple) indicates maximum achievable signal for an individual probe and a value of 10 (pale-green) indicates that 10 % of the total PCR product hybridized to that probe.

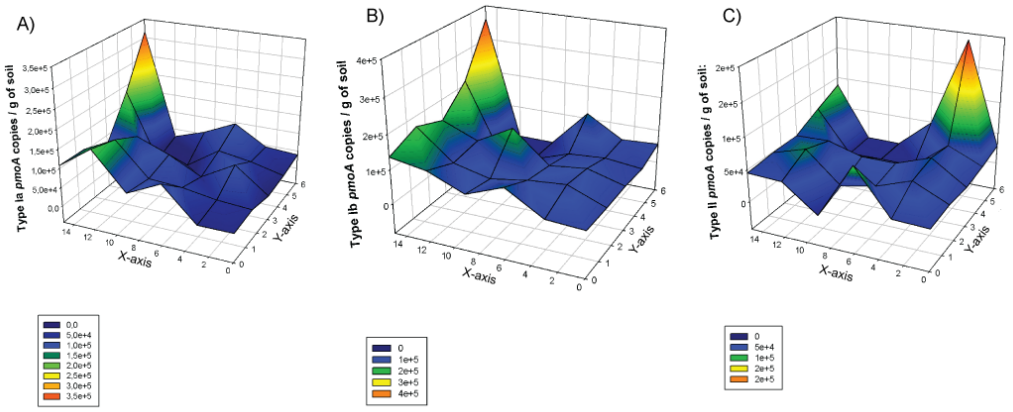


**Fig. S2.** Quantitative PCR results of large scale sampling plot. (**A**) type Ia, (**B**) type Ib and (**C**) type II methanotrophs. Values on xy-axis are meters.

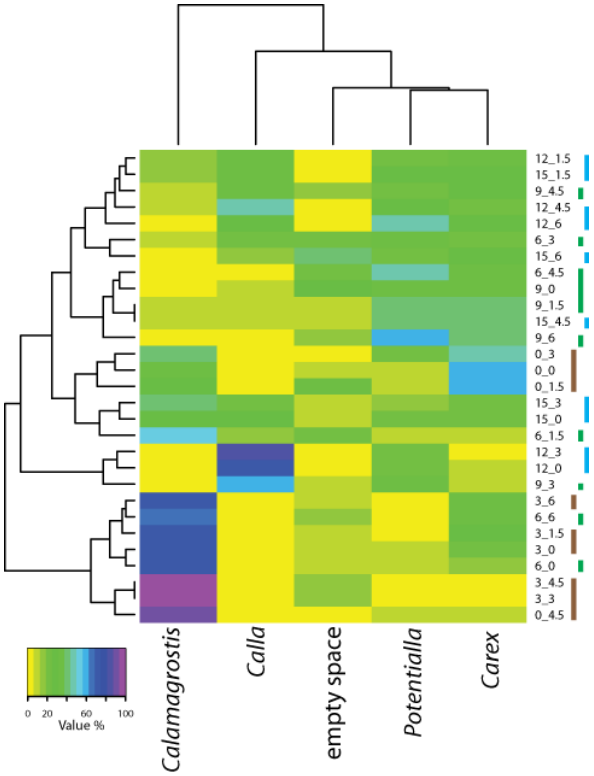


**Fig. S3.** Vegetation over large scale plot (15m x 6m). Above ground plant community was analyzed as coverage percentage of each plant species and empty area (no soil covering plants) around soil sampling site for analysis of spatial heterogeneity of methanotrophs. Name of each sample shows coordinates of sample (x_y see Fig 1a). Color in right sidebar refers to sub-site in littoral wetland; *wet = blue, intermediate = green, dry = brown*.

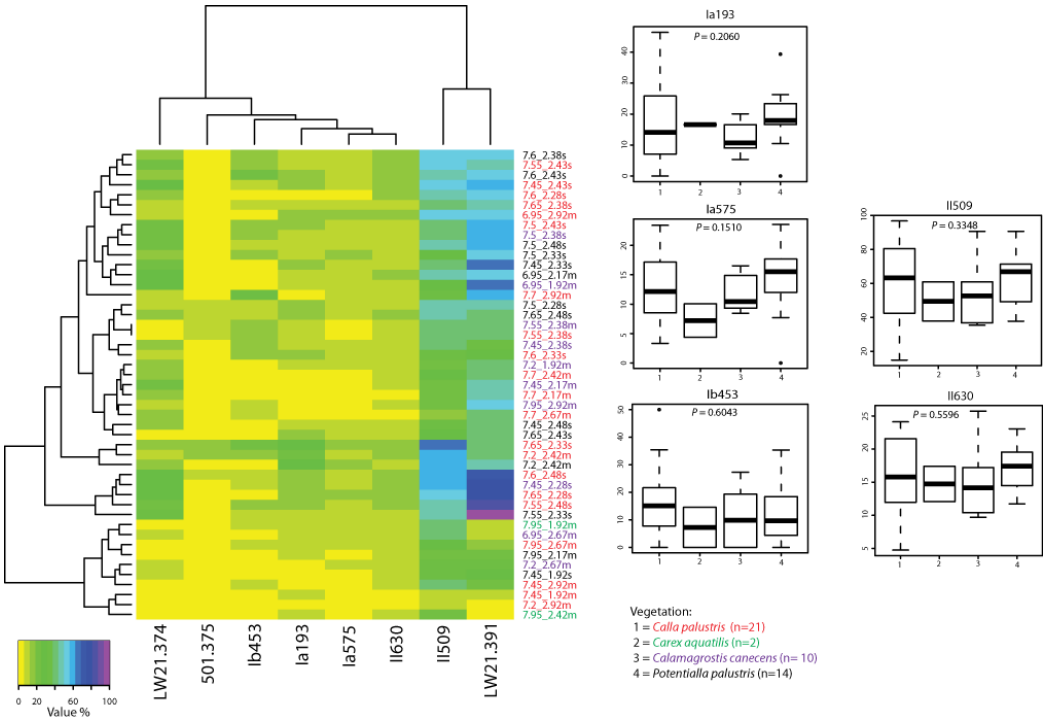


**Fig. S4.** Effect of vegetation on methanotrophs community in medium and small scale sampling plot. Name of each sample shows coordinates of samples (x_y) and plot (medium = m, small = s). Plant species detected in sampling site of soil is shown with color of the sample name. *Calla palustris* (red), *Carex aquatilis* (green), *Calamagrostis canecens* (violet), *Potentialla palustris* (black). Boxplots show universal probe intensities in different vegetation groups inside medium and small sampling plots. Kruskal-Wallis test P-value of vegetation groups is shown in boxplots.

**Fig. S5.** Medium and small sampling plots for study of methanotroph spatial heterogeneity on sampling day July 2006. Approximated location of corners of medium (yellow triangles) and small (red triangles) plots. Sampling locations marked with blue sticks.

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# 5. Succession of methanotrophs in oxygen-methane counter-gradients of flooded rice paddies

ISME Journal (in second revision)

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## 5.1 Abstract

Little is known about population dynamics and contribution of specific taxa to methane oxidation in flooded rice paddies. Here we investigated the succession of methanotrophs in oxygen–methane counter-gradients. We used a gradient microcosm system that simulates oxic–anoxic interfaces of a water-saturated paddy soils, and measured *pmoA*-based (gene encoding particulate methane monooxygenase) terminal restriction fragment length polymorphism (T-RFLP) profiles at both the transcription (mRNA) and the population (DNA) level. The DNA T-RFLP profiles indicated that the methanotrophic community present clearly differed from the active methanotrophic community. We observed a succession of the methanotrophic community over time without any direct effect of pore water chemistry on the community structure. Both the total population and the active subpopulation changed with time, whereas methane oxidation rates remained nearly constant. Hence, we suggest that a diverse microbial seed bank of methanotrophs plays an important role in maintaining the function in a dynamic ecosystem.

## 5.2 Introduction

Atmospheric methane ($CH_4$) is the second most important greenhouse gas after carbon dioxide (Denman et al., 2007). The largest sources are natural wetlands and wetland rice fields, which account for one-third of the annual global methane emissions (Conrad, 2009). In wetland rice fields, aerobic methanotrophs play a key role in controlling methane emission into the atmosphere. In this ecosystem, methanotrophs can reduce the potential methane emissions by 80 % (Conrad and Rothfuss, 1991).

Aerobic methanotrophs are a physiologically distinct group of bacteria that can utilize methane as sole carbon and energy source. Taxonomically they belong to the *Proteobacteria* and *Verrucomicrobia*; methanotrophy in the latter has only recently been discovered (Dunfield et al., 2007; Pol et al., 2007; Islam et al., 2008). Methanotrophs within the *Proteobacteria* are classified into the families *Methylocystaceae* and *Beijerinckiaceae* (type II methanotrophs) and *Methlyococcaeae* (type I methanotrophs) (Bowman, 2006). Type I methanotrophs are further subdivided into types Ia and Ib based on phylogeny (Bodrossy et al., 2003). Type Ia includes many cultivated representatives such as *Methylobacter*, *Methylomonas*, *Methylosarcina*, and uncultivated environmental clones. Type Ib includes besides clusters of uncultivated methanotrophs the genera *Methylococcus* and *Methylocaldum*.

Methanotrophs oxidize methane to carbon dioxide. A key enzyme in this process is the particulate methane monooxygenase (pMMO), which catalyzes the first step. The pMMO is present in all known methanotrophs except the acidophilic *Methylocella* spp. (Theisen et al., 2005). Hence, the *pmoA* gene, which encodes a pMMO subunit, is an excellent molecular marker to identify methanotrophs in environmental samples (McDonald and Murrell, 1997).

DNA-based analyses of protein-coding genes provide information about the total microbial community, including dormant and inactive organisms. RNA-based analyses of protein-coding genes, on the other hand, can elucidate the current *in situ* activity. Different approaches are available to link a biogeochemical function with phylogeny and community structure, e.g., stable-isotope probing (SIP) targeting DNA (Radajewski et al., 2000), rRNA (Manefield et al., 2002), and phospholipid fatty acids (Knief et al., 2003; Shrestha et al., 2008; Bodelier et al., 2009) or transcript-based diagnostic microarrays (Bodrossy et al., 2006). The analysis of protein-coding gene transcripts is a powerful tool for the identification of active bacteria from environmental samples, i.e.,

those bacteria that are actively expressing the gene (Chen et al., 2008). Thus, analyzing the *pmoA* gene and its transcripts should provide a comprehensive profile of the methanotrophic community structure and their activity in the environment, respectively.

When rice paddies are flooded, the bulk soil quickly becomes anoxic, and methane oxidation is restricted to oxic microsites at the soil surface layer and in the rhizosphere (Bosse and Frenzel, 1997). At the soil surface, physical and chemical gradients develop with time. These gradients have a great impact on the activity and structure of the microbial community (Noll et al., 2005). Both type I and type II methanotrophs are present in the soil surface layer (Conrad, 2007; Eller and Frenzel, 2001). It has been proposed that type I methanotrophs are more prevalent when environmental conditions change, and type II methanotrophs are mainly predominant when the environmental conditions are stable (Henckel et al., 2000). However, detailed information about the population dynamics of methanotrophs and the contribution of specific taxa to methane oxidation in nature is lacking.

Here we studied the succession of both present (DNA) and active (mRNA) methanotrophic populations in a model system simulating the oxic–anoxic interface of wetland soils. To link environmental parameters that might determine the population structure, we analyzed environmental soil parameters and correlated them to the methanotrophic community.

## 5.3 Experimental procedure

A microcosm model system was used as described previously (Murase and Frenzel, 2007). Briefly, 14 g of water-saturated rice field soil was filled on top of a PTFE membrane (Whatman, Dassel, Germany) to a height of approximately 3 mm separating a lower from an upper compartment. The lower compartment was initially flushed with nitrogen and then supplemented with methane every two days keeping the concentration at approximately 20 %. Oxygen was supplied to the upper compartment by flushing with filter sterilized air every two days. Methane diffuses from the lower compartment through the membrane into the soil, where methanotrophs formed counter-gradients of methane and oxygen. The formation of counter-gradients was monitored by measuring head space $CH_4$ concentration (Murase and Frenzel, 2007) and soil oxygen microprofiles with a microelectrode (Revsbech, 1989). To study the succession of methanotrophs, a total of 16 microcosms were set up and incubated in the dark at 25°C. At each time point (3, 6, 9, 13, 16, 19, 25, and 30 days), two microcosms were sacrificed. Soil was homogenized, and aliquots were immediately frozen in liquid nitrogen and stored at -80°C for further analysis. To amplify the *pmoA* gene we used the forward primer A189f and the reverse primers A682r (Holmes et al., 1995) or mb661r (Costello and Lidstrom, 1999), hereafter referred to as primer set I and II, respectively. The differences between these primer systems for the Vercelli rice fields are summarized in table S1. We analyzed mRNA and DNA *pmoA*-gene-based terminal restriction fragment length polymorphism (T-RFLP) (Full methods in supplementary information).

## 5.4 Results

The T-RFLP analysis resulted in 14 TRFs that could be affiliated to OTUs of methanotrophs and ammonium-oxidizing bacteria (Table S1). One TRF of 58 bp corresponding *in silico* to sequences representing the RA21-related group was excluded from analysis (Table S1). Cloning and sequencing showed that this particular PCR product was amplified from the rRNA gene of the genus *Myxococcus* having by chance the same length as *pmoA* PCR products. Even removal of 16s rRNA genes with the Epicentre mRNA-ONLY Isolation Kit did not prevent the appearance of unspecific TRFs.

We first compared the total with the active methanotrophic community, combining both primer sets in one correspondence analysis. The analysis depicted a clear separation of DNA and mRNA T-RFLP profiles, and a separation according to primer set I and II (Fig. 1 and S1).

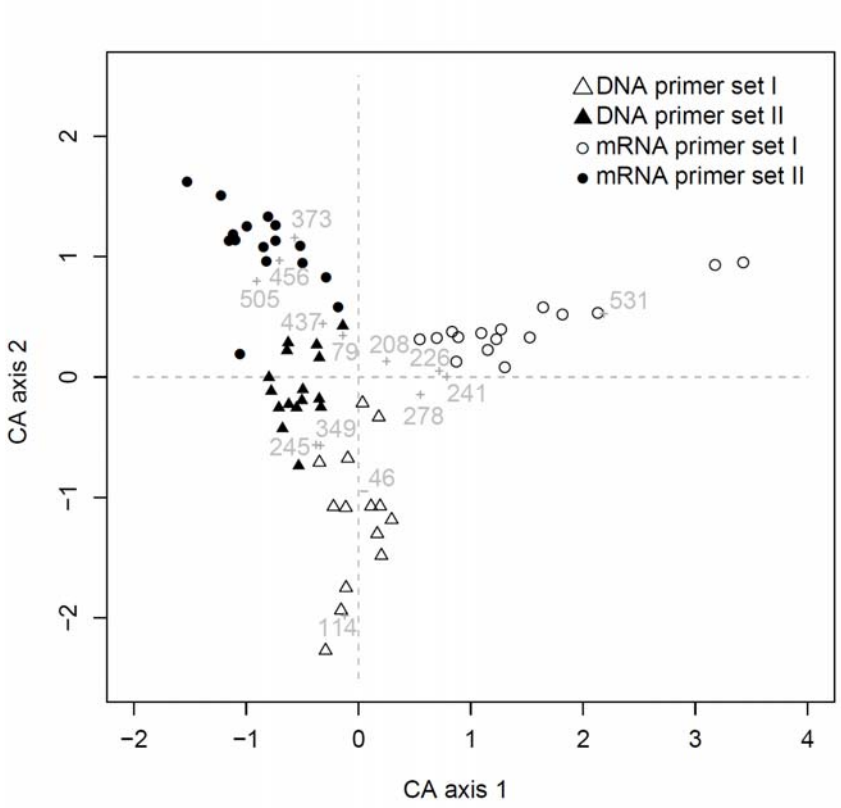


**Fig. 1.** Correspondence analysis (CA) of T-RFLP data at the DNA and mRNA levels with primer sets I and II. The first two axes explained 26.7 and 18.9 % of the total inertia, respectively. Numbers next to species scores depict the size of TRFs representing different phylogenetic units of methanotrophs. The phylogenetic affiliation of the TRFs is given in table S1.

In a second analysis, we considered the primer sets at the DNA and mRNA level separately. We performed a redundancy analysis to identify a succession of methanotrophs. T-RFLP community profiles were significantly affected by time (Fig. 2). Based on this we identified two stages, demonstrating distinct methanotrophic communities, within the first 19 days (stage I) and the last 11 days (stage II) (Fig. 2). No pattern was observed using primer set I at the RNA level. The DNA T-RFLP profiles were characterized by type Ib and type II methanotrophs and ammonium-oxidizing bacteria in the initial stage. The latter stage was characterized by a high dominance of type II methanotrophs (Fig. S1). The mRNA T-RFLP profiles demonstrated a high activity of type Ia and type Ib methanotrophs in the initial stage, and also activity of type II methanotrophs at the latter stage of the succession (Fig. S1).

Finally, the effect of pore water chemistry was analyzed using principal component analysis. We correlated environmental factors to test which environmental parameters control the succession of methanotrophs. We measured nitrate ($NO_3^-$), nitrite ($NO_2^-$), phosphate ($PO_4^{3-}$), sulfate ($SO_4^{2-}$), and ammonium ($NH_4^+$). Only sulfate and ammonium were above the detection limit of 10 µM (Table S2). The ammonium content rapidly decreased within 9 days and then remained stable; the sulfate concentration remained constant for 25 days and then rapidly decreased (Table S2). Several environmental factors were significantly correlated to the principal component analysis (Fig. S2). DNA T-RFLP profiles of primer set I were influenced by ammonium and sulfate ($NH_4^+$: F-value = 0.007; $SO_4^{2-}$: F-Value = 0.006; both $P < 0.001$). The decrease in the ammonium content was correlated with the successional stages (Fig. S2 A). In contrast, DNA T-RFLP profiles of primer set II were not affected by these environmental parameters. mRNA T-RFLP profiles were significantly influenced by sulfate ($SO_4^{2-}$: F-value = 0.005; $P < 0.001$) of primer set II (Fig. S2 B), but profiles of primer set I were not. $CH_4$ oxidation rates remained relatively constant, except on days 3 and 25, when the rate was higher than average (Table S2).

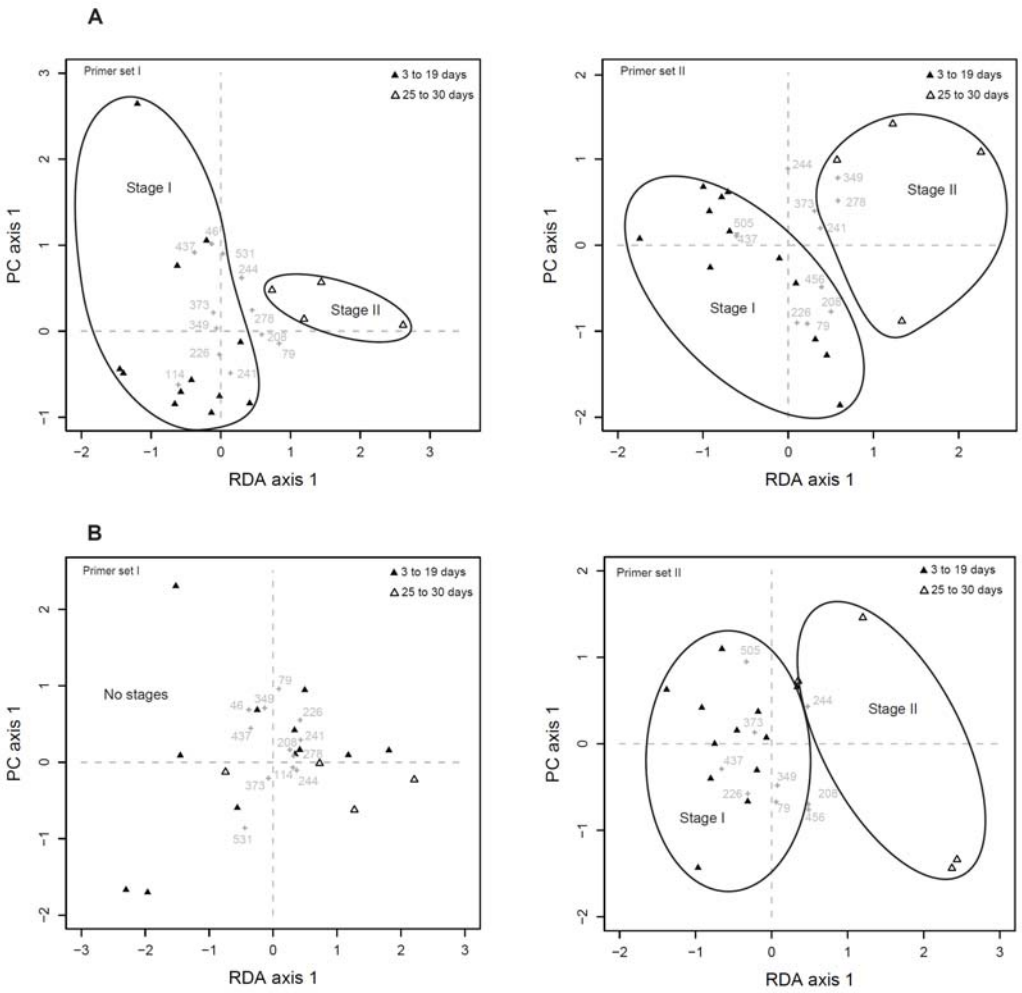


**Fig. 2.** Redundancy analysis of DNA T-RFLP data (A) and mRNA T-RFLP data (B) using primer sets I and II, and with time as the only constraint. The significance of the model was tested by post-hoc ANOVA (primer set I, F = 0.015, $P < 0.01$; primer set II, F = 0.013; $P < 0.01$; RNA: primer set I, F = 0.16; not significant; primer set II, F = 0.072; $P < 0.05$). Different stages of methanotrophic communities are indicated by triangles and are encircled.

## 5.5 Discussion

*The microcosm model system*

The applied microcosm is an excellent model system to study microbial processes at the oxic–anoxic interface of wetland soils because it imitates the natural situation as close as possible (Murase and Frenzel, 2007). Earlier studies used soil slurries or pots with a large amount of soil planted with rice (Eller et al., 2005; Noll et al., 2005; Shrestha et al., 2008). The microcosm used here focuses specifically on the active layer, where methanotrophs thrive in the counter-gradients of methane and oxygen. Hence, the vast number of resting methanotrophs in the bulk soil (Eller and Frenzel, 2001), normally diluting the active community due to the sample size, did not mask actual population changes.

*The applied primer sets*

Methanotrophs can form drought-resistant cysts and exospores (Whittenbury et al., 1970; Bowman et al., 1993) and have been reported to survive unfavorable conditions for up to 170 years (Rothfuss et al., 1997). Hence, differences between the present and the active methanotrophic community might indicate that certain organisms become active during different periods of the season. The abundance of resting stages provides a seed bank from which methanotrophs can become active under optimal environmental conditions (Eller et al., 2005). The succession of methanotrophs observed in our study supports this hypothesis. Primer set II, which best covers the known groups of type I and type II methanotrophs (Bourne et al., 2001), clearly demonstrated a succession of methanotrophs at both the DNA and the mRNA level, specifically of type I methanotrophs (Fig. 2, Fig. S 1). Primer set II also revealed an increase of type II methanotrophs (Fig. S1). In contrast, primer set I revealed an apparent succession of methanotrophs at the DNA level, but not at the mRNA level. This apparent succession of methanotrophs at the DNA level was actually due to ammonium-oxidizing bacteria, which are partially covered by primer set I. In addition, type Ib methanotrophs, represented by a TRF of 79 bp were amplified differently by primer set I and II (Fig. S1). It is known that this TRF represents several clusters/genera of methanotrophs which were not all amplified by both primer sets (Table S1). Hence, different clusters/genera might occur and become active at different stages resulting in different

patterns. Conclusively, the patterns of methanotrophic diversity probably would not have been fully resolved if we had used only one primer set.

*Succession of methanotrophs*

Using *pmoA*-based T-RFLP profiles and multivariate ordination techniques, we demonstrated that the active methanotrophic community clearly differed from the total methanotrophic community (Fig. 1, Fig. S. 1). Analyzing the methanotrophic community at the DNA level, we observed a high relative abundance of type II methanotrophs, which is consistent with earlier work (e.g. Eller and Frenzel, 2001). However, type II methanotrophs did not start to become active until after 25 days (Fig. S1). There is some indication that type Ia methanotrophs require not only methane and oxygen for growth, but also high levels of other nutrients (Bodelier et al., 2000; Noll et al., 2008), whereas type II methanotrophs are less demanding and therefore have an advantage when nutrients are limiting (Graham et al., 1993). Hence, the increase in abundance of type II methanotrophs toward the end of our experiment might be caused by nutrient limitation (Table S2). Several OTUs of type I methanotrophs were detected (Fig. S1). Interestingly, type Ib methanotrophs were both abundant and formed a major group within the active methanotrophic community. Type Ib methanotrophs have been detected as an abundant group in rice fields (Lüke et al., 2009), but their significance, i.e., activity, had not yet been demonstrated. Here we showed that these organisms actively transcribed the *pmoA* gene, which suggests that they contribute greatly to $CH_4$ oxidation in rice fields.

The environmental interpretation of the observed succession of methanotrophs indicated that the recorded soil parameters are not responsible for these patterns (Table S2, Fig. S2). We believe that the decreasing $NH_4^+$ concentration solely influenced ammonium-oxidizing bacteria because $NH_4^+$ was only significant when ammonium-oxidizing bacteria were present (Fig. S2). We assume that the high $SO_4^{2-}$ concentration had only indirect effects on the methanotrophic community.

While direct influences can be correlated easily, indirect influences are rather difficult to link to the community structure, e.g., the effect of protistan grazing (Murase and Frenzel, 2007). In addition, there might be inter- and/or intraspecific competition, e.g., when protistan grazing depletes competitors, thus making new niches accessible for other methanotrophs.

A succession of microbial communities is often explained by the general ecological concept of r- and K-type strategy that was adapted to microbial ecology by Andrew and Harris (1986) (e.g. Noll et al., 2005). Our data indicate that the r- and K-type strategy is not applicable to higher taxonomic levels, because both type I and II methanotrophs contain eco-physiologically very different species. However, single representatives of the traditional type I and II groups may be applicable to the r- and K-type strategy as already suggested (Steenbergh et al., 2009).

*Conclusion*

The microcosm used in our study is an optimal model system for investigating the ecology and function of methanotrophs and simulates the natural conditions of a rice field after flooding as close as possible. The finding of a TRF which derived from ribosomal RNA instead of *pmoA* transcripts emphasizes that phylogenetic assignments in T-RFLP analysis should always be verified by cloning and sequencing. The parallel analysis of mRNA and DNA demonstrated that only a subset of the methanotrophic community present was active at different times. As the population structure changed, methane oxidation rates remained relatively stable. Hence, we suggest that a diverse microbial seed bank together with the functional complementarity of methanotrophs maintains functioning in dynamic ecosystems.

## 5.6 Acknowledgements

We thank the C.R.A.-Agricultural Research Council, Rice Research Unit, s.s. 11 to Torino km 2.5 for support at the rice fields in Vercelli, Italy. We also thank Belinda Schneider and Alexandra Hahn for technical advice. The study was financially supported by a grant from the Deutsche Forschungsgemeinschaft (ESF and EuroDIVERSITY-METHECO).

## 5.7 Supplementary material

*Soil and field site*

Soil samples were collected from a rice field paddy of the C.R.A. Agricultural Research Council, Rice Research Unit, s.s. 11 to Torino km 2.5 (Vercelli, Italy) in autumn 2006 after drainage and harvest. The field site, soil characteristics, and common agricultural practice of the region have been described elsewhere (Holzapfel-Pschorn and Seiler, 1986; Krüger et al., 2001). The soil was air dried and stored at room temperature. Prior to use, the soil was crushed in a jaw crusher (Retsch, Hahn, Germany) and passed through a 2 mm sieve.

*Gas and chemical analyses*

Methane concentration was measured using a gas chromatograph with a flame ionization detector (SRI-8610 A, SRI Instruments, Torrance, Calif., USA). The $CH_4$ oxidation rate was calculated from the balance between two time points. Pore water was sampled by centrifuging water-saturated soil 15 min at 20800 ×*g*. The supernatant was filtered through a 0.2 µm PTFE filter unit and stored at 4°C for further analysis. Ammonium was determined in the supernatant using a fluorometric method as described elsewhere (Murase et al., 2006). The supernatant was also used to measure concentrations of nitrate, nitrite, sulfate, and phosphate by ion chromatography (Bak et al., 1991).

*Nucleic acid extraction, amplification, and T-RFLP analysis*

Prior to extraction, approximately 0.5 g of soil was incubated in a sterile 2 ml Eppendorf tube with 1 ml cold (–80°C) RNAlater ICE (Ambion, Austin, Tex., USA) for 24 h at –20°C and centrifuged at 20800 ×*g* for 5 min. DNA and RNA were simultaneously extracted following the protocol of Lueders and colleagues. (2004). RNA was prepared from 50 µl of nucleic acid extracts by digesting with RQ 1 DNase (Promega, Madison, Wisc, USA) according to the manufacturer's instructions. Digested nucleic acid extracts were purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Nucleic acid extracts were checked by electrophoresis on a 1 % agarose gel. The DNA concentration was quantified using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Del., USA).

cDNA was synthesized and the *pmoA* gene was amplified using the Promega One-step Access RT-PCR System (Promega). For each reaction, 5 µl of RNA was used. Three replicates were carried out per sample. PCR reactions were mixed according to the manufacturer's instructions with the following modifications: 1.5 µl RNasin (Promega), 2.5 µl bovine serum albumin (Roche, Mannheim, Germany), and 1.25 µl DMSO, and were mixed (final volume 25 µl). PCR was carried out with an initial reverse transcription for 45 min at 45°C, and inactivation of reverse transcription and denaturation for 2 min at 94°C, followed by 35 cycles for 30 s at 94°C, 1 min at 55°C, and 1 min at 68°C, and a final elongation for 7 min at 68°C. To check for DNA contamination, a negative control lacked reverse transcriptase. We used the A189f FAM (6- carboxyfluorescein)-labeled forward primer and both A682r (Holmes et al., 1995) and mb661r as a reverse Primer (Costello and Lidstrom, 1999).

DNA was amplified following the same protocol without an initial reverse transcription step. PCR products were checked by electrophoresis on a 1 % agarose gel.

After purification of the PCR products with the GenElute$^{TM}$ PCR clean-up kit (Sigma-Aldrich), purified PCR products were concentrated in an Eppendorf Concentrator 5301 (Eppendorf, Hamburg, Germany) and digested with 10 U of the restriction endonuclease MspI (Fermentas, St. Leon-Rot, Germany) in a total volume of 10 µl for 3 h at 37°C. The mixture was inactivated by heating at 65°C for 20 min. Digested products were purified with SigmaSpin$^{TM}$ post-reaction clean-up columns (Sigma-Aldrich) and centrifuged 5–10 min in a microcentrifuge. Subsequently, 1 µl of each sample was mixed with 0.3 µl MapMarker 1000 (Eurogenetec, Ougree, Belgium) and 11 µl Hi-Di formamide (Applied Biosystems, Foster City, Calif., USA). The samples were denatured for 3 min at 94°C and chilled on ice. T-RFLP analysis was carried out using the GeneScan ABI Prism 3130 (Applied Biosystems). Electropherograms of TRFs between 35 and 600 bp were analyzed using GeneMapper Software Version 4.0 (Applied Biosystems). Peak heights were converted to relative values for further analyses (Lüdemann et al., 2000).

*Statistical analyses of T-RFLP profiles*

We unravelled patterns in the community structure and linked environmental parameters using multivariate ordination techniques. Only TRFs that could be affiliated to methanotrophs and/or ammonium oxidizing bacteria were considered (Table S1).

TRFs of methanotrophs represent genera, clusters, and species. Therefore, each TRF was handled as a methanotrophic operational taxonomic unit (OTU).

Exploratory multivariate ordination techniques display similar samples closer to each other than dissimilar samples. The position of data points in the ordination depicts either the greatest change in abundance (principal component analysis and redundancy analysis), or gives indications about the species composition in a sample (correspondence analysis). While constrained analyses explain the biological variation with the recorded environmental variables, the unconstrained analyses display the dominant pattern of biological variation (Ter Braak, 1986). The appropriate multivariate methods depend on gradient length. Leps and Smilauer (2003) suggest to use linear ordination techniques for gradient lengths $< 3$, unimodal ordination techniques for gradient lengths $> 4$, and either technique for intermediate gradient lengths. We used detrended correspondence analysis (DCA) to identify the gradient length, using either correspondence analysis (CA) for long gradients or principal component analysis (PCA) for short gradients. To check for a succession of methanotrophs, redundancy analysis (RDA) was performed with time as the only constraint. Other linear unimodal and non-metric methods (non-metric multidimensional scaling) were used in parallel to verify the observed patterns. The statistical significance of the environmental parameters was checked using analysis of variance (ANOVA). Pore water chemistry data was standardized to zero mean and unit variance. Environmental data were included in the analyses using vector fitting. All analyses were done with the VEGAN package and the statistical software R (R Development Core Team, 2009; Oksanen, 2009)

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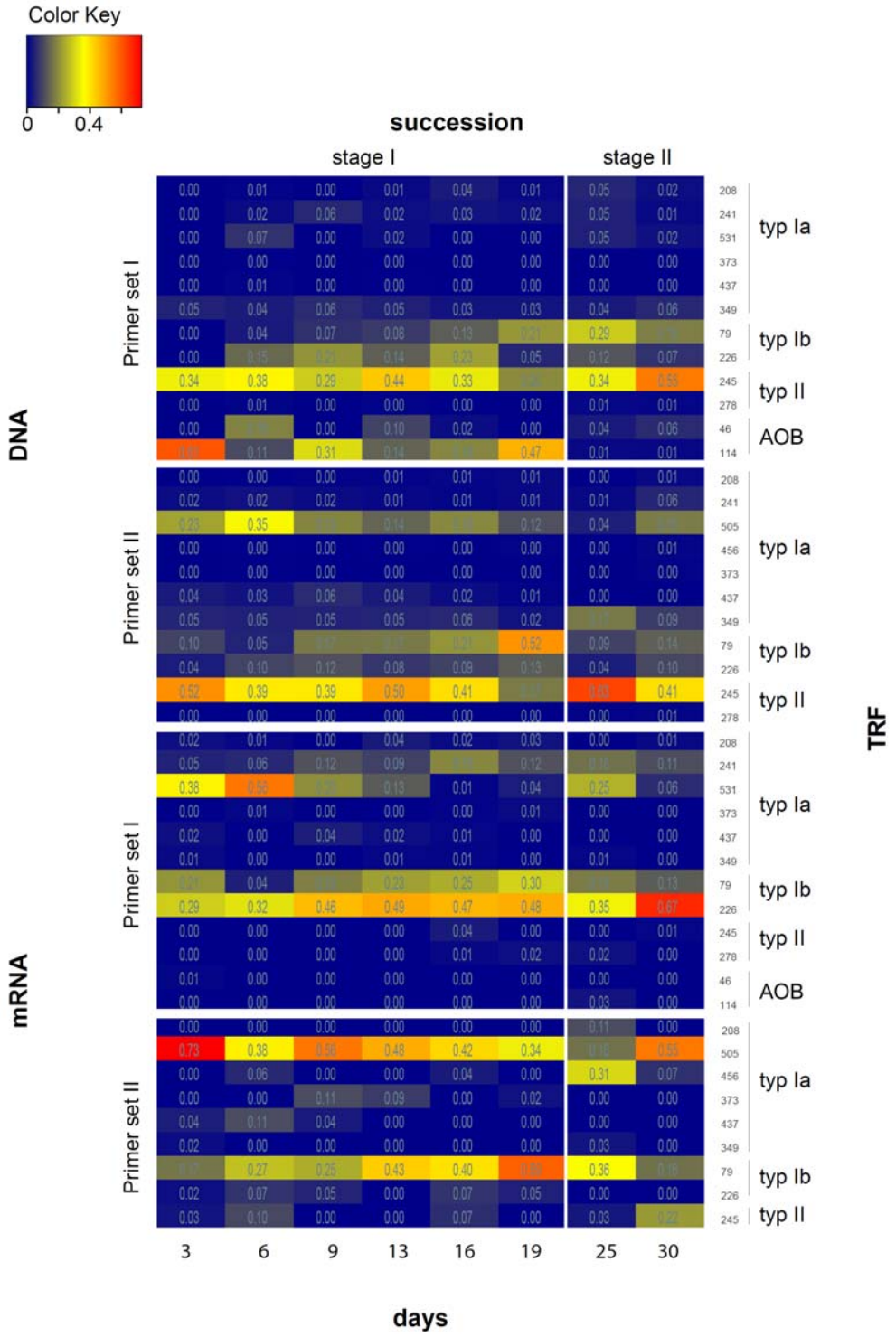


**Fig. S1.** Average values of relative abundances of the present (DNA) and active methanotrophic community (mRNA) detected with primer sets I and II. From bottom to the top the different individual time points are shown, whereas from top to the bottom the two stages of the succession are shown. On the right both TRFs and their affiliation to OTUs of methanotrophs and ammonium oxidizing bacteria are depicted (compare table S1). Color coding bar on the left represents the level of abundance; numbers in the boxes represent the average relative abundances.

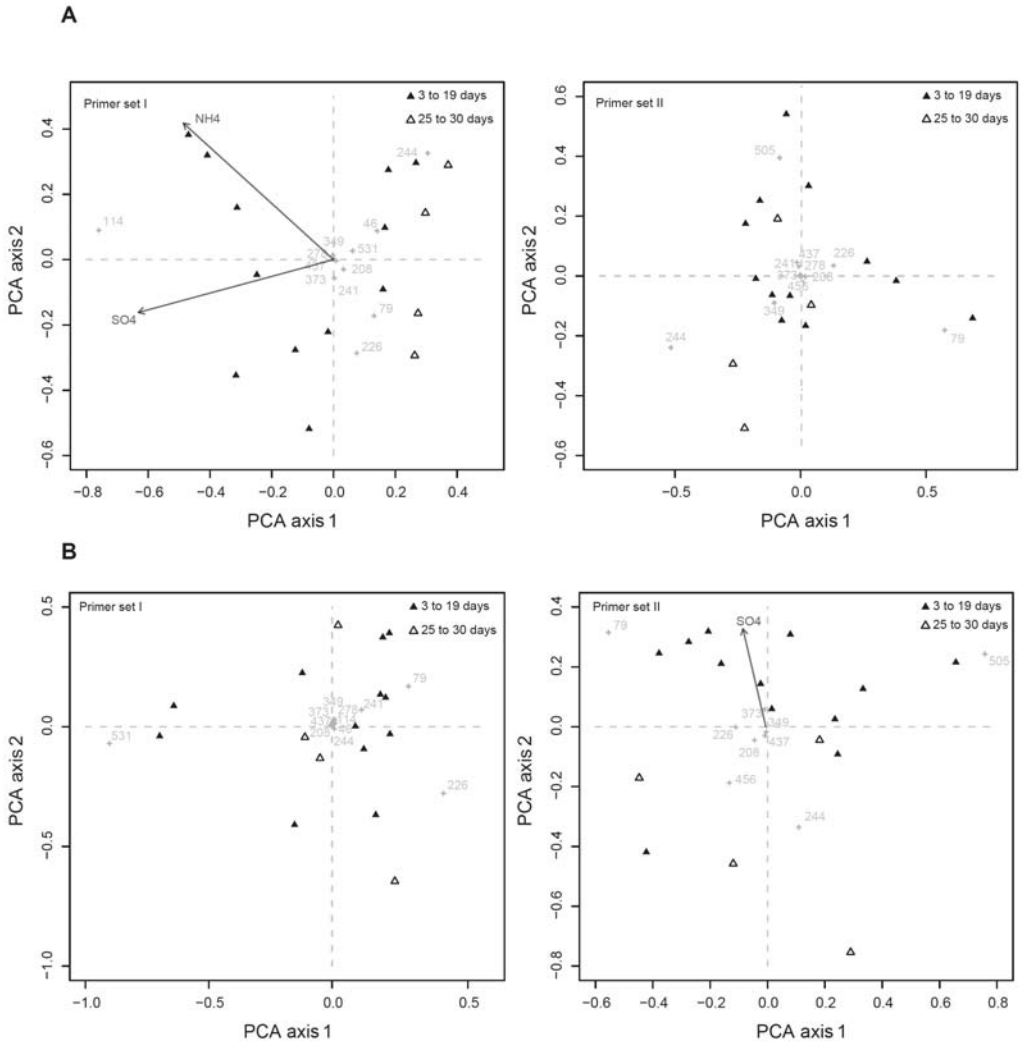


**Fig. S2.** Principal component analysis (PCA) of DNA T-RFLP data (A) and mRNA T-RFLP data (B) using primer sets I and II. The two stages of methanotrophic communities are shown as triangles. Significant environmental factors are drawn as vectors.

**Supplementary table S1.** Phylogenetic inference of *pmoA*/*amoA* sequences from Vercelli rice fields (n = 500) (Lüke et al., 2009) and the detection by the different reverse primers A682r and mb661r. Environmental clusters were named according to representative clones and/or to the denotation of probes as used for a *pmoA* specific diagnostic microarray (Bodrossy et al., 2003; Lüke et al., 2009). Resulting terminal restriction fragments (TRFs) from T-RFLP analysis are shown. TRFs of methanotrophs represent genera, clusters, and species. Therefore, each TRF was handled as a methanotrophic operational taxonomic unit (OTU). TRF size was previously cross-checked by TRFLP analysis of clones.

| **Genus/Cluster** | **Type** | **Reverse primer** | | **OTUs** |
|---|---|---|---|---|
| | | **mB661r** | **A682r** | **TRF length in bp** |
| *Methylomonas* | Ia | + | + | 437 |
| *Methylobacter* LW12/BB5.1 | Ia | + | - | 505, 531 |
| *Methylosarcina fibrata*/*lacus* | Ia | + | - | 208, 241, 349, 437, 456 |
| LP20 | Ia | - | + | 437, 505, 531 |
| *Methylocaldum* | Ib | + | + | 79 |
| *Methylocaldum* related | Ib | + | + | 79, 226 |
| RPC-1 | Ib | + | + | 79 |
| LW21 | Ib[3] | + | - | 79 |
| *Methylocystis/Methylosinus* | II | + | + | 245 |
| MO3 | II | - | + | 33[5] |
| pmoA-2[1] | II | - | + | 278 |
| TUSC | Others[3] | - | + | 33[5], 79 |
| RA21 | Others[3] | - | + | 58 |
| M84-P22 | Others[3] | - | + | 114, 226 |
| M84-P105 | Others[3] | - | + | 33[4], 79, 226,373 |
| Ammonia oxidizers[2] | AOB | - | + | 46, 114 |

[1] The *pmoA*2 is a second copy of the *pmoA* in a single genospecies; it encodes a subunit of a particulate methane monooxygenase (Dunfield et al., 2002). This enzyme possesses different oxidation kinetics (Baani and Liesack, 2008) [2] The *amoA* of ammonium-oxidizing bacteria is homologous to *pmoA* and is partially covered by A682r [3] The sequences cluster between methanotroph *pmoA* and *amoA* sequences from ammonium oxidizers and lack cultivated representatives [4] not detected in this analysis

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**Supplementary table S2.** Pore water chemistry and methane oxidation, mean ± SE, n = 2.

| Days | $SO_4^{2-}$ (mM) | $NH_4^+$ (μmol g dry weight$^{-1}$) | Mean $CH_4$ oxidation (μmol cm$^{-2}$ d$^{-1}$) |
|---|---|---|---|
| 3 | 2.10 ± 0.00 | 4.22 ± 0.13 | 4.36 ± 0.16 |
| 6 | 1.84 ± 0.02 | 1.00 ± 0.13 | 3.55 ± 0.12 |
| 9 | 2.11 ± 0.01 | 0.84 ± 0.10 | 2.98 ± 0.14 |
| 13 | 1.77 ± 0.00 | 0.49 ± 0.02 | 2.84 ± 0.13 |
| 16 | 2.02 ± 0.08 | 0.38 ± 0.02 | 3.24 ± 0.08 |
| 19 | 2.37 ± 0.00 | 0.38 ± 0.01 | 2.85 ± 0.01 |
| 25 | 0.88 ± 0.30 | 0.55 ± 0.00 | 4.11 ± 0.21 |
| 30 | 0.03 ± 0.01 | 0.57 ± 0.12 | 2.46 ± 0.29 |

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# 6. Zusammenfassende Diskussion

$CH_4$ ist neben $CO_2$ einer der wichtigsten Treibhausgase und spielt eine bedeutende Rolle im globalen Klimasystem. Insbesondere zunehmende Erweiterungen und Intensivierungen des Nassreisanbaus haben enorme Auswirkungen auf den globalen Methanhaushalt. In ihrer Funktion als Biofilter oxidieren methanotrophe Bakterien (MOB) in Reisfeldern biogen produziertes $CH_4$ bevor es in die Atmosphäre gelangt und reduzieren die $CH_4$-Emissionen um bis zu 90 %. Obwohl bereits diverse Studien über die Diversität, Physiologie und Ökologie unser Wissen über MOB erweitert haben, fehlen Untersuchungen über räumliche Verteilungsmuster, Zusammenhänge von Diversität und Funktion und die Regulation der MOB durch natürliche und/oder anthropogene Umweltfaktoren. Dieses Wissen ist jedoch wichtig, um die Regulation des globalen Methanhaushalts zu verstehen. Auch der Effekt positver Rückkopplungen kann bisher nicht abgeschätzt werden. So könnten steigende Methanemission in der Atmosphäre zu einem weiteren globalen Temperaturanstieg führen der zu zusätzlichen Methanaustößen in die Atmosphäre führt, wie bereits an auftauenden Permafrostböden gezeigt wurde (Walter et al., 2006).

In der vorliegenden Dissertation wurden in eigenständigen Teilprojekten grundlegende Ergebnisse über die Diversität und Funktion erzielt. Im ersten Projekt wurde der Frage nachgegangen, ob MOB globale und regionale biogeographische Muster aufweisen (Kapitel 2). Ziel des zweiten Projektes war es die räumlichen Strukturierung von MOB am Beispiel des Reisfeldes zu charakterisieren und Konsequenzen für künftige Probenahmen zu ziehen. Im dritten Projekt wurde im Littoral eines borealen Sees als Beispiel für ein natürliches Ökosystem ebenfalls die räumliche Strukturierung methanotropher Bakterien untersucht (Kapitel 3 und 4). Nachdem bisher die räumliche Verteilung im Vordergrund stand wurde in Kapitel 5 in einem Mikrokosmos-Modellystem die zeitliche Entwicklung MOB und der Einfluss von Umweltfaktoren nach der Flutung eines Reisfeldes näher betrachtet. Die Arbeit schließt mit der Frage ab, ob anthropogen oder natürlich Störungen einen Einfluss auf die Stabilität und Widerstandsfähigkeit von methanotrophen Gemeinschaften haben.

## 6.1 Biogeographie methanotropher Bakterien

Eine *pmoA*-Datenbankanalyse (> 4000 Sequenzen) deutet darauf hin, dass es auf globaler Ebene speziell an das Reisfeld adaptierte *pmoA* Genotypen gibt. In Reisfeldern mit gleicher landwirtschaftlicher Praxis in derselben Region können sich jedoch methanotrophe Lebensgemeinschaften deutlich voneinander unterscheiden (Kapitel 2).

Räumliche Verteilungsmuster von Organismen können Aufschluss über die zugrunde liegenden Prozesse geben, die Diversifikation und die Auftrennung in ökologisch unterscheidbare Arten bestimmen. Für die angewandte Ökologie kann das Verständnis und die Vorhersage räumlicher Verteilungsmuster die Grundlage sein, um präzise Ökosystemmanagement-Strategien zu entwickeln (Levin, 1992). Dabei hängt die Identifizierung räumlicher Verteilungsmuster im Wesentlichen von der Wahl der taxonomischen Auflösung ab (Philippot et al., 2009). Auf Ebene der Domäne ist es weitestgehend akzeptiert, dass Bakterien und Archaea ubiquitär verbreitet sind. Betrachtet man allerdings niedrigere taxonomische Ebenen, wie Gattungen oder Spezies, dann zeigen sich sowohl globale als auch endemische Verbreitungsmuster (Ramette and Tiedje, 2007). Der Nachteil einer rein taxonomischen Betrachtungsweise liegt in der schwierigen Interpretation taxonomischer Muster hinsichtlich der Funktion von Population oder Gemeinschaften in ihrer Umwelt, das heißt in ähnlichen Organismen (gleiche 16S rRNA Sequenz) kann eine große Variation ökologischer Funktionen auftreten (Green et al., 2008). Deshalb wird neben einer taxonomischen Betrachtung immer mehr die Untersuchung der Biogeographie an Hand von ökologischen Merkmalen „*Trait-Based Biogeography*“ vorgeschlagen. Diese Betrachtungsweise bezieht sich direkt auf mikrobielle Eigenschaften, die wichtig für die entsprechende Funktion im Ökosystem sind und könnte für die Abschätzung der Folgen einer veränderten Umwelt auf mikrobielle Lebensgemeinschaften von großem Wert sein (Green et al., 2008). Hier eignen sich MOB als ein hervorragendes Modellsystem um mikrobielle Biogeographie zu untersuchen. Zudem kann der Einfluss der Biogeographie auf den globalen Klimahaushalt am Beispiel des $CH_4$ untersucht werden.

## 6.2 Räumliche Verteilungsmuster und Probennahme

Bakterien sind nicht gleichmäßig in ihrer Umwelt verteilt und ihre Aktivität und Abundanz kann sich entlang von Umweltgradienten ändern. Solche Gradienten müssen berücksichtigt werden, wenn Studien über bakterielle Diversität und Funktion konzipiert werden (Franklin and Mills, 2003). Die räumliche Verteilung methanotropher Bakterien (MOB) unterscheidet sich in natürlichen und landwirtschaflich genutzten Flächen. Im Reisfeld wiesen MOB angesichts fehlender Umweltgradienten keine großskalige räumlichen Muster auf (Kapitel 3). Im Gegensatz dazu zeigten sie eine deutliche Strukturierung entlang eines Feuchtegradienten in der littoralen Zone eines borealen Sees (Kapitel 4).

In der mikrobiellen Ökologie werden häufig Experimente mit einer geringen Probenzahl durchgeführt und die Ergebnisse werden genutzt, um auf ganze Ökosystemprozesse zu schließen. Dies ist sehr riskant. Unsere Ergebnisse haben deutlich gezeigt, wie wichtig es ist das Untersuchungsgebiet (Habitat/Ökosystem) genau zu kennen. Am Beispiel der MOB in der littoralen Zone eines borealen Sees lässt sich dies sehr gut veranschaulichen. Der Methanverbrauch und die Zusammensetzung der MOB Gemeinschaften unterschied sich signifikant entlang des Umweltgradienten. Würde man ein Beprobungs-Schema wählen, das den Gradienten nicht vollständig abdeckt, könnten später Schlussfolgerungen gezogen werden, die nicht der realen Situation entsprechen.

Die Ergebnisse aus Kapitel 3 haben Auswirkungen auf Untersuchungen von MOB in Reisfeldern dieser Region. Da kein Umweltgradient berücksichtigt werden muss, können zeitaufwendige und kostenintensive Feldexperimente im kleinen Maßstab im Labor durchgeführt werden. Ein großer Vorteil von Laborexperimenten liegt in der besseren Kontrolle der Umweltbedingungen (zum Beispiel konstante Temperatur), das heißt ein Großteil der Variabilität der Daten, die nicht unmittelbar einen direkten Einfluss auf das Ergebnis haben oder Aufdeckung von Mustern/Strukturen erschweren können, wird ausgeschlossen. Zudem werden a posteriori die Schlussfolgerungen von früheren Laboruntersuchungen (zum Beispiel Krüger et al., 2001) als repräsentativ für diese Reisfelder bestätigt.

## 6.3 Sukzession

*Das r/K- Konzept*

In einem Modellsystem, welches die oxisch-anoxische Grenzschicht von Feuchtgebieten simuliert, wurde eine Sukzession methanotropher Bakterien (MOB) aufgedeckt (Kapitel 5). Es wurde vorgeschlagen, dass mikrobielle Sukzession mit der Verfügbarkeit limitierender Ressourcen und die Ausbeutung dieser durch mikrobielle Lebensgemeinschaften erklärbar sind (Noll et al., 2005). Es basiert auf dem allgemeinen ökologischen r/K-Strategie-Konzept, welches von Andrew und Harris (1986) auf die mikrobielle Ökologie übertragen worden ist. Während r-Strategen schnell auf günstige Lebensbedingungen reagieren und Energie hauptsächlich in die Reproduktion investieren, nutzen K-Strategen ihre Substrate hingegen effektiver und investieren Energie hauptsächlich in einen Erhaltungsstoffwechsel. Dies verschafft ihnen unter limitierenden Bedingungen einen Konkurrenzvorteil.

Das r/K-Konzept als Indikator für mikrobielle Sukzession wurde auch in Versuchen mit Bakterien auf nichtselektiven Medien untersucht. Dabei wurde angenommen, dass die Kultivierbarkeit von Bakterien als Stellvertreter für Opportunismus steht, das heißt für schnelles Wachstum unter geeigneten Bedingungen, welches ein typisches Merkmal der r-Strategen ist. Das Verhältnis von opportunistischen Zellen zu der Gesamtzellzahl war am Anfang höher als zu einem späteren Zeitpunkt. Dies deutet auf einen Übergang von Zellwachstum zu einem Erhaltungsstoffwechsel hin (Garland et al., 2001). Diese Annahme wird weiter dadurch gestützt, dass die Kopienzahl des rRNA Operons (rrn) Hinweise auf ökologische Strategien gibt. Auf einem Komplexmedium formten Bakterien mit einer Kopienzahl > 4 rrn früher Kolonien, als Bakterien mit einer Kopienzahlen < 2 rrn (Klappenbach et al., 2000). Dies konnte auch für eine mikrobielle Sukzession im Reisfeldboden gezeigt werden. Hier enthielten Isolate in der frühen Phase der Sukzession mehr rrn Kopienzahlen, als Isolate in der späten Phase (Shrestha et al., 2007). In der frühen Phase der Sukzession dominieren r-Strategen und in späteren Stadien der Sukzession werden sie durch K-Strategen ersetzt.

In der vorliegenden Arbeit begannen Typ I MOB sofort zu wachsen, während Typ II MOB zwar die ganze Zeit detektiert, jedoch nur unter limitierenden Bedingungen aktiv wurden. Auf der anderen Seite koexistierten Typ I und II MOB am Ende des Experiments (Abb. 1). Deshalb können Typ II MOB sowohl den K-Strategen (Abb. 1,

Phase I) als auch den r-Strategen (Abb. 1, Phase II) zugeordnet werden, je nach dem, welche Phase betrachtet wird.

Die Ergebnisse weisen auf ein grundsätzliches Problem innerhalb der MOB hin. Die ökologische Differenzierung von Typ I und II ist nicht mehr sinnvoll, denn diese Gruppen enthalten ökophysiologisch sehr unterschiedliche Arten. In Bezug auf das r/K-Konzept bedeutet es, dass dieser Ansatz im Großen nicht anwendbar ist, jedoch für die ökologische Differenzierung einzelner Vertreter der Typ I und II MOB sinnvoll sein kann (zum Beispiel Steenbergh et al., 2009). Möglicherweise sind beobachteten Trends hinsichtlich der ökologischen Differenzierung zwischen Typ I und II sogar ökosystemspezifisch (Knief et al., 2006).

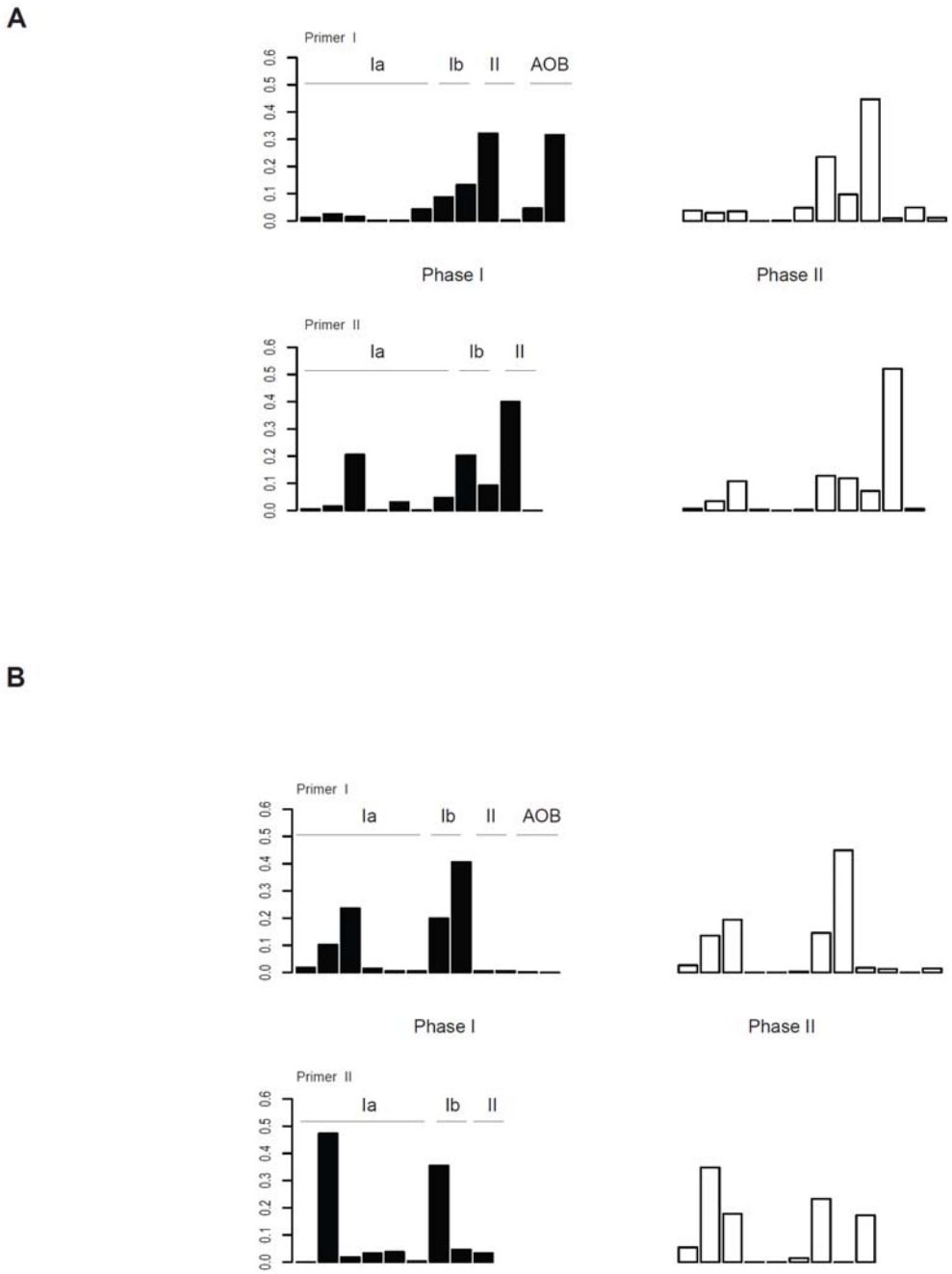


**Abb. 1.** Gemittelte relative Abundanzen der gesamten (A) und der aktiven (B) methanotrophen Gemeinschaft amplifizert mit den Reverse Primern I (A682r) und II (mb661r). Es werden die zwei Phasen der Sukzession gezeigt. Die einzelnen Balken im Histogramm repräsentieren verschiedene Vertreter der einzelnen Untergruppen methanotropher Bakterien. Nähere Informationen sind in Kapitel 4 zu finden.

*Aktivität und Präsenz methanotropher Bakterien*

Es wurde sowohl die gesamte (DNA) als auch die aktive (mRNA) Population methanotropher Bakterien (MOB) untersucht. Dabei wurde Typ Ib als eine abundante Gruppe in Reisfeldern nachgewiesen (Kapitel 2). Zudem konnte gezeigt werden, dass Typ Ib aktiv das *pmoA* Gen transkribiert haben, was eine Beteiligung an der Methanoxidation in Reisfeldern nahelegt (Kapitel 5). Diese Entdeckung ist besonders interessant vor dem Hintergrund, dass das repräsentative TRF (79bp) auch die Gruppe des Rice Paddy Cluster I abdeckt. Die Sequenzen im Rice Paddy Cluster I gehören vermutlich zu MOB, die speziell an das Reisfeld adaptiert sind (Kapitel 2).

In einer Studie von Chen und Kollegen (2008) über die Diversität von MOB in einem Moor wurde gezeigt, dass Typ II MOB sowohl die gesamte als auch die aktive Population dominierten. Im Experiment aus Kapitel 3 stellten Typ II die größte Gruppe MOB in der Gesamtpopulation dar, wiesen aber nur eine geringe Aktivität auf, die im Laufe der Zeit zunahm (Abb. 1 B).

Man geht davon aus, dass mRNA Transkripte Protein-kodierender Gene nur in aktiven Organismen zu finden sind (Wawer et al., 1997). Bei methanotrophen Bakterien gibt es gibt Hinweise darauf, dass sich der Anteil an *pmoA* mRNA Transkripten zwischen Typ I und II unterscheidet (Steenbergh et al., 2009). Inwiefern die mRNA mit der Aktivität gekoppelt ist oder ob dieser Unterschied vielleicht verschiedene ökologische Strategien darstellt ist bisher nicht vollständig geklärt.

*Umweltfaktoren*

Die Interpretation der beobachteten Sukzession mit Hilfe der Umweltdaten ergab keinen Zusammenhang mit den gemessenen Bodenparametern (Kapitel 4). Auch wenn die gemessenen abiotischen Faktoren keinen Einfluss auf die methanotrophen Bakterien (MOB) hatten, können durchaus Umweltfaktoren vorliegen, welche für die beobachteten Muster verantwortlich sind, zum Beispiel Kupfer (Semrau et al., 2010). Zudem zeigte in Kapitel 3 eine Rangkorrelation nach Spearman, dass Typ I und II (MOB) je nach betrachteter OTU positiv oder negativ miteinander korreliert sein konnten, obwohl im Reisfeld die Lebensgemeinschaft MOB durch keinen großskaligen Umweltgradienten strukturiert wurde. Eine mögliche Erklärung für die beobachteten Muster stellt der Einfluss von biotischen Faktoren dar, zum Beispiel Prädation. Die Prädation von Bodenbakterien durch räuberische Protisten ist ein verbreitetes Phänomen (Murase et al., 2006; Murase and Frenzel, 2007). Für MOB konnte gezeigt werden, dass einzellige Protozoen nur bestimmte MOB als Beute bevorzugen (Murase and Frenzel, 2008). In einem Folgeexperiment mit einem hochauflösenden *pmoA* spezifischen Microarray wurde nachgewiesen, dass Typ Ia MOB besonders häufig als Beute bevorzugt wurden, während es keine Prädation gegenüber Typ II gab. Eine Prädation der Typ II durch einige Protozoen ist jedoch möglich. Für Typ Ib konnten keine Aussagen gemacht werden, da diese im untersuchten Habitat unabhängig vom Prädationsdruck nicht dominant vorkamen (Murase pers., Mitteilung).

## 6.4 Stabilität der methanotrophen Lebensgemeinschaften

In Kapitel 5 wurde vorgeschlagen, dass die Diversität methanotropher Bakterien (MOB) in Form einer „seed bank“ im Reisfeld für die Aufrechterhaltung ihrer Funktion eine wichtige Rolle spielt. Studien haben gezeigt, dass die Methanoxidation durch Veränderung der Umwelt, zum Beispiel Düngung (Conrad and Rothfuss, 1991; Bodelier et al., 2000a; Bodelier et al., 2000b;) beeinflusst wird. Nun stellt sich die Frage, wie widerstandsfähig und belastbar die Diversität MOB gegenüber solchen Störungen ist und welche Auswirkungen dies auf den globalen Methanhaushalt hat. In einem abschließenden Experiment wurde der Einfluss der Düngung auf die methanotrophen Lebensgemeinschaften unter einem niedrigen und hohen $CH_4$ Fluss untersucht. Die vorläufige Auswertung zeigt, dass nur die unterschiedlichen Methanflüsse einen signifikanten Effekt auf die methanotrophe Lebensgemeinschaften haben (Abb. 2). Also

scheint der Effekt des Methanflusses eine größere Bedeutung für die methanotrophe Lebensgemeinschaft zu haben als der Einfluss des Ammoniums. Zudem ergab eine Dufrene-Legendre Indikator Spezies Analyse (Dufrene and Legendre, 1997), dass die Gattung *Methylobacter* und MOB deren *pmoA* Sequenzen zum Rice Paddy Cluster 2 gehören, indikativ für die unterschiedlichen Methanflüsse sind. Diese MOB sind vermutlich an Habitate mit hohen Methankonzentrationen adaptiert, da sie nur unter hohem Methanfluss in großer relativer Abundanz detektiert wurden.

In einem anderen Experiment gab es bereits deutliche Hinweise darauf, dass MOB sehr effektiv neuen Lebensraum besiedeln, der durch eine gravierende Störung mit einer hohen Mortalitätsrate der vorherrschenden Mikroorganismen entstehen kann (Ho et al., 2010). Die Diversität der MOB nahm in gestörten Proben ab, während die $CH_4$ Oxidationsraten jedoch konstant blieben oder sogar anstiegen. Dies sind aber bisher die einzigen Studien dieser Art und weitere müssen durchgeführt werden. um allgemeingültige Aussagen zu treffen.

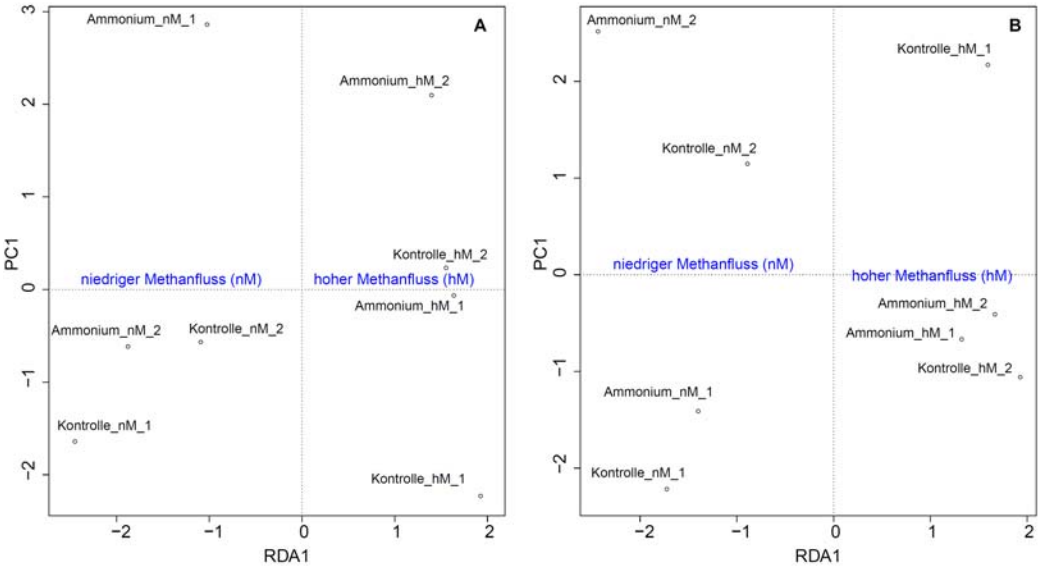


**Abb. 2.** Redundanzanalyse der *pmoA* spezifischen Microarray Daten (DNA) für beide Reverse Primer (**A**) mb661r und (**B**) A682r. Die grundlegenden Unterschiede der beiden Primer Paare sind in Kapitel 5 beschrieben. Vor der statistischen Analyse wurden die Microarray Daten standardisiert (Lüke et al., 2010). Es sind nur die signifikanten Ergebnisse dargestellt. Die Signifikanz wurde mit einer post-hoc ANOVA getestet: **(A)** F = 3.2884 und (**B**) F = 2.1276, $P < 0.005$. Das Model erklärt 35 % (**A**) bzw. 26 % (**B**) der Variabilität. Als Modelsystem diente der Mikrokosmos (Kapitel 5). Für jeden $CH_4$ Fluss (niedriger Methanfluss = 2 % Methankonzentration; hoher Methanfluss = 20 % Methankonzentration) wurden zwei Mikrokosmen mit je einem Replikat angesetzt, wie in Kapitel 5 beschrieben. Nach 14 Tagen wurde einerseits Wasser als Kontrolle, anderseits 80 mM di-Ammoniumsulfatlösung zu den Mikrokosmen hinzugegeben. Die Konzentration, welche eine Hemmung der MOB bewirkt, wurde in einem Vorversuch experimentell bestimmt.

## 6.5 Schlussbetrachtung und Ausblick

Die durchgeführten Arbeiten in dieser Dissertation liefern wichtige Erkenntnisse zur Diversität und Funktion methanotropher Bakterien (MOB). Dabei wurden in diesem Zusammenhang auch grundlegende methodische Fragen beantwortet.

Methanotrophe Lebensgemeinschaften weisen biogeographische Muster auf und eignen sich als gutes Modellsystem, um biogeographische Fragestellungen zu beantworten. Umweltgradienten haben einen Effekt auf die Lebensgemeinschaften der MOB und müssen bei der Planung von Experimenten berücksichtigt werden, um repräsentative Aussagen über die zu untersuchenden Prozesse machen zu können. Man muss sowohl die gesamte (DNA) als auch die aktive (mRNA) Lebensgemeinschaft der MOB betrachten, um ein umfassendes Bild der vorliegenden Dynamiken zu bekommen. Dabei scheint die Diversität in Form einer „seed bank" eine große Rolle für die Erhaltung ihrer Funktion zu haben. Unterschiedliche Energieflüsse scheinen einen größeren Effekt auf die methanotrophen Lebensgemeinschaften zu haben als kurzfristige Störungen wie zum Beispiel Stickstoffdüngung. Schließlich hat sich gezeigt, dass die ökologische Differenzierung von Typ I und II MOB sich weitaus schwieriger gestaltet und vermutlich eine weitaus feinere Auftrennung innerhalb der MOB notwendig ist. Auch eine Ökosystemspezifität scheint möglich. Diese Dissertation zeigt aber auch, dass es noch viele offene Fragen zur Diversität, Ökosystemfunktion und ihrer Regulation gibt.

Diese Arbeit war Bestandteil des europäischen Forschungsprojektes METHECO „*The role of microbial diversity in the dynamics and stability of global methane consumption: microbial methane oxidation as a model-system for microbial ecology*". In diesem Projekt wurde ein *pmoA* spezifischer Microarray (Bodrossy et al., 2003; Bodrossy et al., 2006) mit einer Fülle neuer Sequenzen methanotropher Bakterien aus verschiedenen Habitaten ergänzt. In zukünftigen Studien bietet die Anwendung dieses Microarrays eine gute Grundlage zur Analyse der methanotrophen Diversität (DNA) und Aktivität (mRNA). In Kombination mit dem „stable isotope probing" der DNA (Radajewski et al., 2000), rRNA (Manefield et al., 2002) und/oder Phospholipid-Fettsäuren (Bodelier et al., 2009) lassen sich zudem direkte Zusammenhänge der Diversität mit ihrer Funktion untersuchen. Außerdem erlaubt der *pmoA* spezifische Microarray vergleichende Studien in unterschiedlichen Habitaten, die bisher angesichts

der unterschiedlichen Methoden zur Erfassung der MOB nicht möglich waren. Schließlich bietet die Entwicklung neuer Sequenzierungsmethoden (zum Beispiel Pyrosequencing) eine weitere Möglichkeit um detaillierte Studien der methanotrophen Diversität durchzuführen und vielleicht kann so auch die Bedeutung der „rare biosphere“ (siehe Kapitel 2) entschlüsselt werden.

Fundierte Theorien existieren nur in der Pflanzen- und Tierökologie, die auf die mikrobielle Ökologie übertragen werden, zum Beispiel das r/K-Konzept (Andrews and Harris, 1986). Mikro- und höhere Organismen weisen jedoch große Unterschiede auf, die die Anwendung solcher Konzepte limitieren (Prosser et al., 2007). Höhere Organismen können in ihrer Umwelt mit großer Sicherheit erfasst, kategorisiert und studiert werden. Bei Mikroorganismen ist das weitaus schwieriger, denn sie weisen oft kaum morphologische Unterschiede auf und der Großteil lässt sich nicht oder nur schwer kultivieren (Sait et al., 2002). Zudem können bei Mikroorganismen in nahe verwandten Arten (gleiche 16S rRNA Sequenz) eine große Variation ökologischer Funktionen auftreten (Green et al., 2008). Der Gebrauch mikrobieller Modellsysteme, zum Beispiel des in dieser Arbeit verwendete Mikrokosmos`s (Kapitel 4), können genutzt werden, um ökologische Theorien zu testen. Denn ein sehr komplexes natürliches System wird imitiert, während die räumliche und zeitliche Heterogenität reduziert wird. Durch die gewonnenen Erkenntnisse aus dem METHECO Projekt und ihrer definierten Funktion eignen sich MOB als hervorragendes Modellsystem, um solchen Fragestellung nachzukommen.

Unsere Ergebnisse weisen darauf hin, dass durch Änderungen der Umweltbedingungen die Diversität der MOB in Form einer „seed bank“ eine wichtige Rolle für die Aufrechterhaltung der Funktion hat. In folgenden Studien muss die Widerstandsfähigkeit und Belastbarkeit der methanotrophen Diversität auf Umweltstörungen näher untersucht werden. Denn welche kurz- oder langfristigen Auswirkungen diese Veränderungen auf den globalen Methanhaushalt haben, kann bisher nicht abgeschätzt werden.

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# 7 Anhang

## Lebenslauf

Sascha Krause geboren am 06.06.1980 in Nordhorn

**Schulbildung:**

| | |
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| 1987 – 1991 | Grundschule Matthias-Claudius-Schule Lingen (Ems) |
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**Hochschulstudium:**

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| 2001 – 2006 | Studium der Biologie am Fachbereich Biologie/Chemie der Universität Osnabrück<br>Bachelor of Science in Biologie (Biologie der Organismen)<br>Titel der Bachelor Arbeit: Habitat preferences of Cepero's groundhopper, *Tetrix ceperoi* (BOLÍVAR, 1887) (2004).<br>Master of Science in Biologie (Biologie der Organismen)<br>Titel der Master Arbeit: Population trends of Orthoptera in northwestern Germany (2006). |

**Praktikum:**

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| 2006 – 2007 | Praktikum bei Prof. Dr. Peter Frenzel am Max-Planck-Institut für Terrestrische Mikrobiologie in Marburg |

**Promotion:**

| | |
|---|---|
| 2007 – 2010 | Promotionsstudium am Fachbereich Biologie der Phillips-Universität Marburg<br><br>Doktorarbeit bei Prof. Dr. Peter Frenzel am Max-Planck-Institut für Terrestrische Mikrobiologie in Marburg und Mitglied der International Max Planck Research School (IMPRS) |

## Beiträge zu wissenschaftlichen Tagungen (nur Promotion)

Krause, S. and Frenzel P. (2007). Spatial heterogeneity of methanotrophs: a geostatistical analysis of pmoA-based T-RFLP patterns in a paddy soil. First EuroDiversity annual conference, Oktober 2007, Paris, Frankreich (Poster).

Krause, S. and Frenzel P. (2008). Spatial heterogeneity of methanotrophs: a geostatistical analysis of pmoA-based T-RFLP patterns in a paddy soil. VAAM Jahrestagung, März 2008, Frankfurt, Deutschland (Poster).

Krause, S. and Frenzel, P. (2009). Microbial ecology: Structure and functioning of methanotrophs in a flooded rice field soil. Internal METHECO meeting, Januar 2009, Nieuwersluis, Niederlande (Vortrag).

Krause, S. and Frenzel, P. (2009). Microbial ecology at the microcosm level: Activity and population dynamics of methanotrophic bacteria during early succession in a flooded rice field soil. VAAM Jahrestagung, März 2009, Bochum, Deutschland (Poster).

Krause, S. and Frenzel, P. (2009). Microbial ecology on the microcosm level: Activity and population dynamics of methanotrophic bacteria during early succession in a flooded rice field soil. European Geosciences Union General Assembly, April 2009, Wien, Österreich (Vortrag).

Krause, S. and Frenzel, P. (2009). Microbial ecology on the microcosm level: Activity and population dynamics of methanotrophic bacteria during early succession in a flooded rice field soil. BAGECO 10 conference, Juni 2009, Uppsala, Schweden (Poster).

Krause, S. and Frenzel, P. (2009). Activity and composition of the methanotrophic community in a flooded rice field soil. Internal METHECO meeting, September 2009, Abisko, Schweden (Vortrag).

## Workshops

ESF Workshop “Microbial diversity and ecosystem functioning”. März 2007, Wassercluster Lunz, Österreich.

Training in Bioinformatik: Training in Linux and ARB/SILVA, mit Vorlesungen, Übungen und Seminaren. Februar 2008, Bremen, Deutschland.

ESF Workshop “Metacommunities: Spatial dynamics and ecological communities”. Mai 2008, Uppsala, Schweden.

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## Abgrenzung der Eigenleistung

Die Arbeit ist Bestandteil des europäischen Forschungsprojektes METHECO „The role of microbial diversity in the dynamics and stability of global methane consumption: microbial methane oxidation as a model-system for microbial ecology". Die Finanzierung des Projekts erfolgte durch die Deutsche Forschungsgemeinschaft (DFG) und der europäischen Forschungsgemeinschaft (ESF). Die Eckpfeiler meiner Arbeit sind von meinem Betreuer Prof. Dr. Peter Frenzel konzipiert worden.

Soweit nicht anders erwähnt, wurden alle Experimente von mir selbst geplant und durchgeführt, sowie anschließend in Form eines Manuskriptes ausgewertet. Das abschließende Verfassen der Manuskripte erfolgte zusammen mit meinem Betreuer Prof. Dr. Peter Frenzel.

Kapitel 4 besteht aus einem Artikel, der zusammen mit Kollegen aus dem METHECO Projekt bearbeitet wurde. Ein Teil der geostatistischen Analyse wurde von mir übernommen. Zudem habe ich Anregungen zur Struktur und Inhalt der Diskussion gegeben. Das endgültige Erstellen des Manuskripts erfolgte durch Henri Siljanen (Universität Kuopio, Finnland).

Kapitel 2 besteht aus einem Artikel, der zusammen mit meiner Arbeitskollegin Claudia Lüke und meinem Betreuer Prof. Dr. Peter Frenzel erstellt wurde. Das endgültige Erstellen des Manuskripts erfolgte durch Claudia Lüke.

## Erklärung

Ich versichere, dass ich meine Dissertation

**Ökologie methanotropher Bakterien:**

Räumliche Verteilung und Funktion methanotropher Bakterien in Feuchtgebieten

selbständig und ohne unerlaubte Hilfe angefertigt habe und mich keiner als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe. Diese Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Marburg, März 2010